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# *Nitric oxide and neuropeptides in the gut:*
## *Changes in ulcerative colitis, pouchitis and short bowel syndrome*

by

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**Academic Dissertation**

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*All you need is guts*

To my family

## *CONTENTS*

## ABSTRACT

The aim of this study was to examine how inflammation and adaptation change expression of neurotransmitters in the enteric nervous system. Immunohistochemical techniques have been applied to reveal specific neurotransmitter and neuropeptide systems of the human and pig intestine. GAP-43 (growth- associated protein-43), PGP 9.5 (protein gene product 9.5), NSE (neuron specific enolase) and synaptophysin were evaluated as general neuronal markers in the human gut by using computerized morphometric analysis. Changes in neuropeptide-containing innervation caused by ulcerative colitis were examined in patient specimens. The expression of the three nitric oxide synthase (NOS) isoforms in ulcerative colitis colon and the expression of NOS-2 and NOS- 3 in pouchitis were examined. The specimens were harvested in operations of ulcerative colitis patients and the control specimens from patients operated for colon tumors. Ileum reservoir biopsies were taken during endoscopy. Changes in neuropeptide-containing innervation after massive proximal small bowel resection during adaptation were investigated quantitatively in the pig.

GAP-43 turned out to be a universal neuronal marker in the mature human intestine. It reveals more numerous and thicker nerve fibers than PGP 9.5, synaptophysin or NSE. In contrast, NSE is a superior marker of neuronal somata. Ulcerative colitis colon does not significantly change the total number of nerve fibers in the colon. However, the density of substance P-containing nerve terminals specifically increases in the mucosa. NOS-1 disappears selectively from the nerves of muscularis mucosae of ulcerative colitis colon. NOS-2 level increases in the epithelium of ulcerative colitis colon in relation to the severity of the disease. Ulcerative colitis colon causes an increase in the number of vascular profiles, and a relative decrease in the level of NOS-3 in the lamina propria. NOS-2 is induced in pouchitis and correlates with both the clinical degree of pouchitis and with the severity of acute inflammation. NOS-3 immunoreactive vascular profiles increase in pouchitis. The present series of studies demonstrate that substance P and NO may play substantial roles in the pathogenesis of ulcerative colitis and pouchitis. Massive resection induces significant changes in the neuropeptide-containing innervation in the intestinal mucosa and muscle layer. The changes are specific for VIP, galanin and enkephalin and are compatible with altered motor activity and mucosal function in the remaining intestine.

## *LIST OF ORIGINAL PUBLICATIONS*

This thesis is based on the following original publications, which will be referred to by their Roman numerals.

I. Vento P, Soinila S. Quantitative comparison of growth associated protein-43, neuron specific enolase and protein gene product 9.5 as neuronal markers in mature human intestine. The Journal of Histochemistry & Cytochemistry 47:1405-1415, 1999

II. Vento P, Kiviluoto T, Keränen U, Järvinen HJ, Kivilaakso E, Soinila S. Quantitative comparison of growth associated protein- 43 and substance P in ulcerative colitis. In press The Journal of Histochemistry & Cytochemistry, 2001

III. Vento P, Kiviluoto T, Järvinen HJ, Soinila S. Changes in distribution of three isoforms of nitric oxide synthase in ulcerative colitis. Scandinavian Journal of Gastroenterology 36:180-189, 2001

IV. Vento P, Kiviluoto T, Järvinen HJ, Kärkkäinen P, Kivilaakso E, Soinila S. Expression of inducible and endothelial nitric oxide synthases in pouchitis. Inflammatory bowel diseases 7:120-127, 2001

V. Vento P, Kiviluoto T, Pakarinen M, Lauronen J, Halttunen J, Kivilaakso E, Soinila S: Nerve terminals containing neuropeptides decrease in number after massive proximal small bowel resection in the piglet. Digestive Disease and Science 43:1102-1110, 1998

## ABBREVATIONS

| | |
|---|---|
| ABC | Avidin-biotin-complex-staining |
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| CCK | Cholecystokinin |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanine monophosphate |
| CGRP | Calcitonin gene-related peptide |
| ENS | Enteric nervous system |
| GABA | γ-aminobutyric acid |
| GAP-43 | Growth-associated protein -43 |
| GRP | Gastrin-releasing peptide |
| 5-HT | 5-hydroxytryptamine |
| IPAA | Ileal pouch-anal anastomosis |
| NKA | Neurokinin A |
| NKB | Neurokinin B |
| NOS | Nitric oxide synthase |
| NOS-1 (nNOS) | Neuronal nitric oxide synthase |
| NOS-2 (iNOS) | Inducible nitric oxide synthase |
| NOS-3 (eNOS) | Endothelial nitric oxide synthase |
| NPY | Neuropeptide Y |
| NSE | Neuron specific enolase |
| PARS | PolyADP-ribose synthetase |
| PBS | Phosphate buffered saline |
| PGP 9.5 | Protein gene product 9.5 |
| SEM | Standard error of the mean |
| UC | Ulcerative colitis |
| VIP | Vasoactive intestinal polypeptide |

## *INTRODUCTION*

The gut is innervated by the enteric nervous system, which controls the motility, exocrine and endocrine secretion, microcirculation and is also involved in regulation of immune and inflammatory processes [1].

The pattern of innervation can be conveniently examined in full thickness biopsy specimens of the intestine. Classical histological methods, such as the silver staining [2] can be used to reveal neurons or nerve fibers, although these techniques are tedious to perform, they are nonspecific and they do not allow immunohistochemical colocalization studies. By using immunohistochemical techniques we can observe specifically neurotransmitters and -peptides in neurons and nerve fibers [3]. Examination of specific neuronal markers, antigens expressed exclusively by the nervous tissue, allows us to estimate changes involving the gut innervation as a whole.

Neuron-specific enolase (NSE) and protein gene product 9.5 (PGP 9.5) are widely used neuronal markers in the human gut [4-6]. Synaptophysin is a structural component of synaptic vesicles, but it has been used as a marker of neurons as well [7]. Recently it has been shown that GAP-43 is abundantly expressed in the autonomic neurons and nerve fibers, as well as in the enteric nervous system [8; 9] of the adult rat. The specificity and sensitivity of these markers in the human gut is not known. In the study I we evaluate GAP-43 as a general neuronal marker in the mature human gut, its colocalization with neuropeptides, and its usefulness in computerized morphometric analysis of the gut innervation in comparison with two other neuronal markers, PGP 9.5 and NSE.

Nitric oxide (NO) is both a physiological messenger molecule and a cytotoxic agent. It has many physiological functions in the intestine. It is a neurotransmitter; it regulates the circulation and participates in defense mechanisms [10-13].NO is produced by three different isoforms of the nitric oxide synthase (NOS). A constitutive nitric oxide synthase is present in neurons (NOS-1)[14; 15]. The function of NO produced by NOS-1 is relaxation of the smooth muscle [16-18]. Another constitutive NOS isoform in ubiquitously localized in the vascular endothelium (NOS-3) [13].

Ulcerative colitis (UC) is an inflammatory, ulcerating process in the mucosa of colon. The precise etiology is unknown, but it is obvious that a number of mediators and cytokines are responsible for many of the clinical manifestations of UC [19]. Changes in innervation have been reported in inflammatory bowel disease [20-24]. Previous studies by our group have revealed increased number of substance P-immunoreactive nerve fibers in the mucosa of UC colon [25]. In study II we examine the changes in the innervation of UC colon to find out how specific the increase in the density of innervation is for substance P. NOS-2 expression is induced in inflammatory bowel diseases including UC [26]. It has

remained unknown whether NOS-1 or NOS-3 undergoes changes in UC. In study III we investigate expression of all three isoforms of NOS in UC.

Abdominal colectomy with ileal pouch-anal anastomosis (IPAA) has become the surgical treatment of choice for most patients with UC. The most frequent long-term complication of IPAA performed on UC patients is inflammation of the ileal reservoir, termed "pouchitis"[27; 28]. In study IV we examine whether NO production is induced in pouchitis, whether it varies in different clinical forms of pouchitis and whether it correlates with the histopathological changes of pouchitis.

After massive small bowel resection, the remaining intestine undergoes both morphological and functional changes [29-32]. During this process, called adaptation, secretory and absorptive functions as well as motility of the intestine change. The study V was performed to reveal changes occurring in neuropeptide innervation pattern after massive small bowel resection.

# *REVIEW OF THE LITERATURE*

## NITRIC OXIDE

### *Synthesis and properties*

Nitric oxide (NO) is one of the smallest molecules in nature. In 1987-88 it was discovered that vascular endothelial cells are able to synthesize NO from L-arginine [33; 34]. Following studies revealed that NO can be synthesized by many mammalian cells and it modulates immune function, blood vessel dilatation, and neurotransmission [35].

Arginine is converted to citrulline by nitric oxide synthase (NOS) in a two-step enzyme reaction via the formation of the intermediate Nω-hydroksy-L-arginine and finally release of NO. NOS enzyme exists in three isoforms encoded by distinct genes [35].

Types 1 (nNOS) and 3 (eNOS) are constitutive and present in the neural tissue and in the vascular endothelium, respectively. NOS-1 and NOS-3 are regulated at post-translational level by calmodulin via $Ca^{++}$-dependent mechanism [35]. Type 2 NOS (iNOS) is $Ca^{++}$-independent and is induced by bacterial endotoxins and cytokines in macrophages, endothelium, smooth muscle, liver, fibroblast, and neutrophils [35; 36] . Activity of NOS-1 and NOS-3 produces low levels of NO for a short period of time. NOS -2 when induced provides a continuous supply of high levels of NO.

NO is uncharged and it diffuses freely across cell membranes. In biological systems its half-life is less than 30 s. NO is a less reactive than many free radicals and it cannot react with itself [37].

NO mediates its effects as a physiological messenger via production of cGMP by activating guanylate cyclase [38]. Interactions of NO with thiol groups may also provide a mechanism whereby NO can be transported to the target cell [37]. Nitrosylation of thiol proteins may also be involved in remodeling of axon terminals [39]. Under conditions of oxidative stress, e.g. when high levels of NO are synthesized by NOS-2 and intracellular levels of superoxides are high, the intracellular thiol pool is depleted. NO can react with superoxide ($O_2^-$) to produce peroxynitrite ($ONOO^-$) and subsequently the hydroxyl radical, which are both more toxic than NO itself [37]. Peroxynitrite is a highly toxic substance oxidizing a variety of molecules and triggering cytotoxic processes including lipid peroxidation and DNA damage [40]. NO can inhibit enzyme activity be reacting with Fe-S groups or R-SH groups. Nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) results in irreversible ADP-ribosylation [37]. NO can also cause deamination of DNA resulting in damage that activates polyADP-ribose synthetase (PARS). These interactions cause cytostasis, energy depletion, mutagenesis and ultimately cell death.[11; 12] (Figure1).

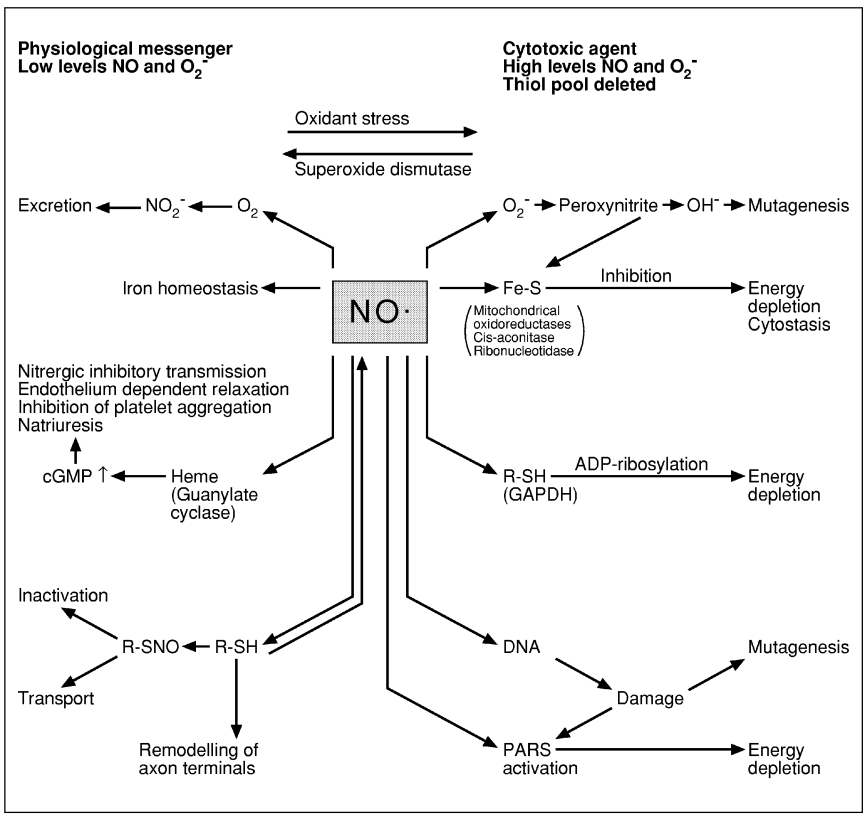


**Figure 1.** Schematic representation of reactions and effects of NO. NO can act as a physiological messenger and as a cytotoxic agent. Under normal conditions, low levels of NO are synthesized by NOS-1 and NOS-3 and intracellular levels of superoxides are low. NO mediates many of its physiological effects by increasing the production of cGMP. Under condition of oxidative stress high levels of NO are synthesized by NOS-2 and intracellular levels of superoxides are high. NO can inhibit enzyme activity and finally cause energy depletion and cytostasis. Modified from Lincoln et al 1997 [37].

### *NOS-1 and intestine*

NOS-1 is localized in enteric ganglia and their process [14; 41; 42]. The function of NO produced by NOS-1 is relaxation of smooth muscle. In experimental animals NOS-1 mediates muscle relaxation through inhibitory junction potential [16; 17; 43]. NOS-1 has been reported to co-localize with VIP in human colon [15; 44].

***NOS-2 and intestine***

NOS-2 has not been demonstrated in the normal human intestine. However NOS-2 expression is induced in inflammatory bowel disease [26], intestinal infections [45], and in celiac disease [46].

***NOS-3 and intestine***

NOS-3 is localized in the vascular endothelium. NO produced by NOS-3 is responsible for vasodilatation through relaxation of the vascular smooth muscle, and it contributes to regulation of blood fluidity and prevention of platelet aggregation [13]. Experimental evidence suggests that NOS-3 activity is regulated by bloodborn agonists activating endothelial receptors, shear stress or low oxygen tension [47; 48].

## ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) represents one of the three divisions of the autonomic nervous system. The ENS is composed of several nerve plexuses in the different wall layers of the gut and of their interconnections [49].

The enteric nervous system is primarily derived from cells of the vagal segment of the neural crest that migrate to the cranial portion of the gut and subsequently move caudally to populate the gastrointestinal tract. The ganglia of the hindgut receive an additional contribution from the sacral segment of neural crest [50].

Meissner (1857) and Auerbach (1864) first described the rich neuronal network composed of ganglia and nerve bundles forming the submucous and the myenteric plexuses in the gut wall. Bayliss and Starling (1899) provided further evidence for this network, which is not only the target of various "extrinsic" pathways, projecting via the sympathetic and parasympathetic divisions of the autonomic nervous system, but also contains an exceedingly high number of intramural neurons [51]. It is this "intrinsic" nerve network, running uninterruptedly from the oral cavity to the anal canal, which Langley (1921) called "the enteric nervous system".

The majority of nerve cell bodies of the ENS are confined to the ganglia of submucous (Meissner) and myenteric (Auerbach) plexuses and constitute the intrinsic component of the enteric nervous system. The myenteric plexus is located between the two muscle layers, the inner circular and the outer longitudinal one. The submucous plexus is located between the muscularis mucosae and the circular muscle layer. A sparse subserous plexus is found in the mesentery and on the outside of the muscle layer. Mucosal plexus is comprised of nerve fibers in lamina propria [52].

The neurons that make up the enteric nervous system can be classified as intrinsic afferent neurons (sensory), interneurons, and motor neurons. The intrinsic afferent neurons form the sensory limb of all intrinsic motor and

secretomotor reflex pathways and they are located in both the myenteric and submucous plexuses. Interneurons connect the intrinsic afferent neurons with the motor or secretomotor neurons. Interneurons involved in motor reflexes are directed orally or anally, and called ascending or descending, respectively. The motor neurons are either excitatory or inhibitory. The excitatory motor neurons project locally or orally to the circular muscle layer, and their neurotransmitters are acetylcholine and substance P. The inhibitory motor neurons in the circular muscle layer project anally and contain vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) [49; 53; 54].

In addition to entirely intrinsic neurons, the gastrointestinal wall contains extrinsic nerve fibers, which can be divided in motor (efferent) and sensory (afferent) pathways of the parasympathetic and sympathetic divisions. The parasympathetic motor pathways consist of branches of the vagus nerve that control the motor and secretomotor functions of the upper gastrointestinal tract and of the sacral parasympathetic nerves that regulate the functions of the distal colon and rectum. The parasympathetic efferent neurons are all cholinergic and have an excitatory effect on the myenteric neurons. The sympathetic efferent fibers entering the gut are postganglionic fibers with cell bodies in the prevertebral ganglia. They are noradrenergic and they innervate secretomotor neurons, presynaptic cholinergic nerve endings, submucosal blood vessels and gastrointestinal sphincters. Primary afferent neurons are sensory neurons that carry information from the gut to the central nervous system. Their fibers are located in the vagal and splanchnic nerves. The primary vagal afferent nerve endings in the muscle layer are sensitive to mechanical distension of the gut, while those in the mucosa are sensitive to luminal concentration of glucose, amino acids, or long-chain fatty acids, some reacting to a wide variety of chemical and mechanical stimuli. The cell bodies of the vagal primary afferent neurons are located in the nodose ganglia. Splanchnic primary afferent neurons have their cell bodies in the dorsal root ganglia. They are nociceptors and are involved in sensing pain in the gastrointestinal tract. They respond to high-intensity mechanical or chemical stimuli that damage or threaten the tissue [53-55]. These neurons contain substance P and also CGRP [56]. Substance P and CGRP may be important in the activation of nociceptive afferent neurons in conditions such as noncardiac chest pain, colon irritabile syndrome, intestinal ischemia, and inflammatory bowel disease [54]. Splanchnic primary afferent neurons can also act directly on the nearby effector systems. They have long, bifurcated processes that allow them to induce the axon reflex. In this reflex the activation of one limb of the bifurcated axon causes excitation to spread to the collateral limb, which then releases neurotransmitters such as substance P to produce effects on the nearby cells [57]. The axon reflex is important in mucosal vasodilatation, duodenal secretion, and mast-cell degranulation [54; 58-60].

This intrinsic nervous network is capable of selecting and initiating highly coordinated functional responses, while the connections and projections from/to the other parts of the nervous system have a modulatory role [61]. Most diverse digestive functions are regulated by the enteric nervous system,

including, motility, many secretory and absorptive processes, mesenteric [62] as well as local vasomotor responses [63] and mucosal defense [61].

Changes in the density of innervation are caused by various pathological processes, such as ulcerative colitis [25], irradiation [64],Chagas´s disease [65], achalasia [66; 67], Hirschsprung´s disease [68], or intestinal neuronal dysplasia [69].

***Neurotransmitters***

More than 20 putative neurotransmitters have been found in the intestine either by immunohistochemistry, radioimmunoassay or bioassay (Table 1) [49]. The evidence that peptides are located in intestinal neurons and nerves relies on immunohistochemical studies [70]. Most intestinal neurons contain several neurotransmitters, and distinctive patterns of transmitter colocalization have been observed [71-73]. Neurotransmitter functions have been defined only for acetylcholine, substance P, vasoactive intestinal polypeptide, and nitric oxide [54]. A wide variety of neurons that have different functions may use the same neurotransmitter [73].

*Substance P*

Substance P was the first of the gut neuropeptides. In 1931 von Euler and Gaddum reported that extracts of horse intestine contained material that stimulated atropine-resistant contractions of rabbit ileum [74]. 1970 substance P was isolated from hypothalamic tissue and chemically characterized as an undecapeptide, and was shown to have potent vasodilator properties [75]. Substance P occurs widely in the brain and spinal cord as well as in the gut [70]. Substance P belongs to the tackykinins, which are a family of small biologically active peptides whose principal mammalian members are substance P, neurokinin A (NKA) and neurokinin B (NKB). Substance P and NKA are in abundance in the digestive system. These peptides are derived from precursor proteins, the preprotachykinins, which are coded by two different genes. Tachykinins act through specific tachykinin receptors [76].

Substance P is expressed in both the intrinsic and the extrinsic neurons [71; 72; 76]. The latter are mainly located in the dorsal root ganglia [77]. The intrinsic afferent nerves located in the submucous plexus contain, in addition to substance P, also acetylcholine or dynorphin. The intrinsic afferent nerves located in the myenteric plexus contain substance P and calbindin. Stimulation of these neurons releases substance P in the enteric ganglia, which activates secretomotor pathways, but also within the mucosa to directly influence epithelial function [56]. The intrinsic nerves may be responsible for triggering enteric reflexes to luminal stimuli [70]. The primary afferent nerve fibers contain substance P and CGRP (calcitonin-gene-related peptide [78]. There is relatively little change in substance P concentrations in the gut in capsaicin-pretreated animals (capsaicin depletes substance P storage granules in extrinsic sensory nerve terminals of the gut). This indicates that the major pool is intrinsic neurons (figure 2) [56]. Excitatory motor neurons contain both substance P and acetylcholine. Substance P may act both on the enteric neurons and on the

muscle and it controls intestinal peristalsis by contracting intestinal smooth muscle and by stimulating acetylcholine release from other nerves [76].

Tachykinins have a role in mediating the vasodilatation and extravasation associated with neurogenic inflammation in skin, joints and airways [79-81]. Substance P also participates in inflammatory reactions in the gut [25; 82-84].

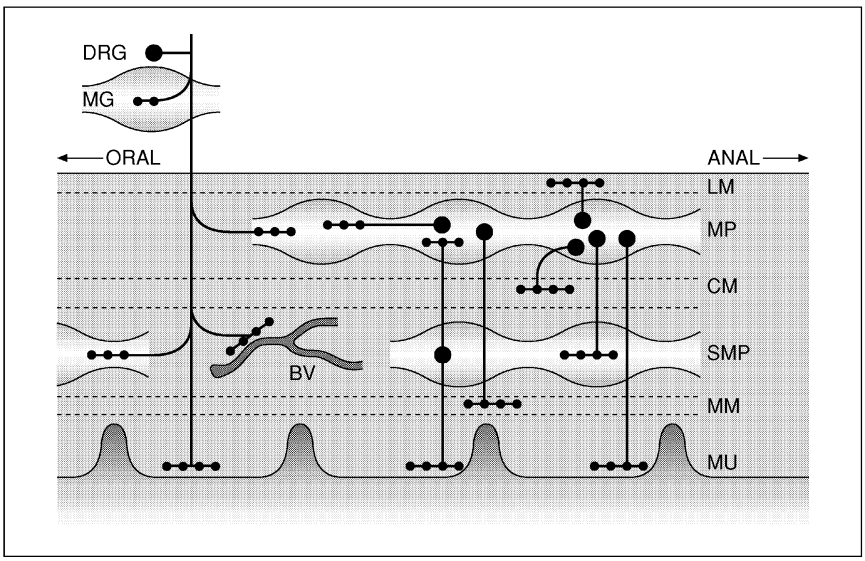


**Figure 2.** Schematic diagram of projections of substance P-immunoreactive neurons in the enteric nervous system. DRG, dorsal root ganglion; MG, mesenteric prevertebral ganglion; MP myenteric plexus; SMP submucous plexus; LM, longitudinal muscle; CM, circular muscle; MM, muscularis mucosae; MU mucosa (modified from Holzer and Holzer-Petsche 1997[76]).

VIP

Neurons containing VIP are commonly found throughout the mammalian gastrointestinal tract [70]. In the human, VIP has been found in all layers of the intestine [42]. VIP relaxes vascular and nonvascular smooth muscle and is an effective stimulant of electrolyte secretion from the intestine and pancreas [70].

The neurons responsible for the descending inhibition of peristalsis in the circular muscle layer contain VIP and NO [70; 85].

Galanin

Galanin is present in secretomotor neurons and descending interneurons as well as in some inhibitory motor neurons of the human intestine[86].

Enkephalin

Enkephalin and related opioid peptides are present in interneurons and motor neurons. In most regions these substances probably provide feedback inhibition of transmitter release and inhibit intestinal motility. Opiates have potent antisecretory effects in small intestine, which is thought to be the mechanism of the antidiarrheal actions of these compounds [86].

Somatostatin

No clearly defined role has been described for somatostatin in spite of its widespread distribution in the enteric neurons [86].

Calcitonin gene-related peptide

CGRP is present in some secretomotor neurons and in some interneurons. It is also colocalized with substance P in the extrinsic sensory neurons [70].

Acetylcholine

Acetylcholine is the most abundant transmitter substance in the gut. It is the primary excitatory transmitter of the muscle, intestinal epithelium, some gut endocrine cells and it also acts at neuroneuronal synapses [86].

Noradrenaline

Noradrenaline is located in the postganglionic sympathetic terminals and it inhibits motility in non-sphincter regions, contracts the smooth muscle of the sphincters, inhibits secretomotor reflexes and is also vasoconstrictor [86].

NO

Nitric oxide is a co-transmitter in enteric inhibitory neurons [15-17; 43; 85]. It is also a possible transmitter at neuroneuronal synapses. It colocalized with VIP [44].

Additionally, other mediators, such as ATP, CCK, angiotensin, dynorphin, GABA, GRP, 5-HT, neuromedin U, and NPY have been described in the gastrointestinal tract [70; 86].

**Table 1. Putative neurotransmitters in the mammalian enteric nervous**

| | |
|---|---|
| Acetylcholine | The most common transmitter in the gut. Present in motor, secretomotor and interneurons Primary excitatory transmitter to the muscle, to the epithelium, to some gut endocrine cells, and at neuroneuronal synapses. |
| AMINES | |
| Noradrenaline | Noradrenergic nerve fibers in the intestine are extrinsic. Primary transmitter of sympathetic neurons. Inhibit motility in non-sphincter regions; contract the muscle of the sphincters; inhibit secretomotor reflexes, act as a vasoconstrictor neurons to the enteric arterioles. Separate neurons control each function. |
| Serotonin | Appears to participate in excitatory neuroneuronal transmission |
| AMINO ACIDS | |
| Gamma-aminobutyric acid | Present in different population of neurons, depending on species and region. Does not appear to be a primary transmitter |
| PURINES | |
| ATP | Probably contributes to transmission from enteric inhibitory muscle motor neurons |
| PEPTIDES | |
| Substance P | Present in intrinsic and extrinsic sensory neurons, intrinsic motor neurons. Excitatory transmitter to smooth muscle (co-transmitter with Ach), may contribute to excitatory neuroneuronal transmission from enteric primary sensory neurons. |
| VIP | Excitatory transmitter of secretomotor neurons, contributes to transmission from enteric inhibitory motor neurons (co-transmitter with NO), possibly a transmitter of enteric vasodilatator neurons. |
| Galanin | Present in secretomotor neurons and descending interneurons, in some inhibitory motor neurons. Roles unknown. |
| Somatostatin | No clearly defined role |
| CGRP | Present in some secretomotor neurons, interneurons, in axons of primary sensory neurons. Role unknown. |
| Cholecystokinin | Present in some secretomotor neurons and in some interneurons. May contribute to the excitatory transmission. |
| Neuropeptide Y | Present in secretomotor neurons; appears to inhibit secretion of water and electrolytes. Also present in interneurons and some inhibitory motor neurons. Probably not a primary transmitter |

| | |
|---|---|
| Neuromedin U | A role as a transmitter is not established |
| Opioids | |
| Enkephalin | Present in interneurons and motor neurons. Probably provides feedback inhibition of transmitter release, prejunctional inhibition |
| Dynorphin | In secretomotor neurons, interneurons and some motor neurons. Does not appear a primary transmitter. |
| Endorphins | |
| Peptide YY | |
| Pituitary adenyl cyclase-activating peptide | Localized predominantly in myenteric ganglia and smooth muscle |
| Thyreotropin-releasing Hormone | |
| Vasoactive intestinal contractor (an endothelin) | |
| GASES | |
| Nitric oxide | In inhibitory motor neurons (co-transmitter with VIP). |
| Carbon monoxide | |

modified from McConalogue and Furness, Coyal and Hirano [54; 86]

## NEURONAL MARKERS

Changes in the density of innervation can be observed when examined in full thickness biopsy specimens of the intestine. Apart from immunohistochemical techniques revealing a particular neurotransmitter or neuropeptide, it is useful to estimate changes involving the gut innervation as a whole. Classical histological methods, such as the silver staining [2] used to reveal neurons or nerve fibers are nonspecific and they involve treatments that are not compatible with immunohistochemical colocalization studies. A number of neural proteins, i.e. proteins originally isolated, or characterized, from nerve tissue, mainly brain, have been studied with respect to their putative exclusive presence in the enteric nervous system. Neurofilaments are neuron-specific intermediate filaments. They consist of three different polypeptide subunits and are an integral component of the cytoskeleton. However, not all neurons show neurofilament protein immunoreactivity [87]. The presence of neurofilaments in the myenteric and submucous plexi has been reported, however mucosal nerves in the villi of the lamina propria are neurofilament protein-deficient both in animals [88] and human (Soinila, personal communication). S-100 protein, originally isolated from the brain, has been localized in the gut, in both Schwann cells and enteric glial cells [89]. Extensive studies have revealed the presence of S-100 protein immunoreactivity in many non-nervous sites [6].

***Growth-associated protein-43***

The 43 kD growth-associated protein (GAP-43) is an acidic membrane-bound phosphoprotein, expressed in conditions of embryonic growth, during axon regeneration and even at maturity in certain areas of the brain known to exhibit synaptic plasticity [90-93]. GAP-43 induces process outgrowth from neurons [94]. In the adult peripheral nervous system GAP-43 expression is generally low. However, expression of GAP-43 increases heavily after injury [95]. Sprouting, uninjured neurons also express GAP-43 [96]. Moreover, denervation of motor endplates results in GAP-43 upregulation by terminal Schwann cells [97]. GAP-43 has been described in the rat stomach, and small and large intestine [8; 88; 98; 99]. GAP-43-immunoreactive nerves have also been reported in the human small intestine and rectum [9; 69; 100]. VIP has been found to co-localize with GAP-43 in the ferret ileum [9]. In the rat jejunal villi, all electron microscopically identifiable nerve profiles were found to be GAP-43-positive [88]. No further studies of co-localization of GAP-43 and neurotransmitters have been reported.

***Protein gene product 9.5***

Protein gene product (PGP) 9.5 is a cytoplasmic protein, specific for neurons and cells of the diffuse neuroendocrine system [5; 101-103]. It has been used in various contexts to reveal nerve cells and fibers. It has been claimed to be sensitive neuronal marker, even better than NSE, in whole-mount preparations of mammalian intestine [104; 105]. Comparison of GAP-43 and PGP 9.5 has shown that only a fraction of nerve fibers in normal adult pancreas exhibits GAP-43 immunoreactivity, whereas most if not all nerve fibers are PGP 9.5-immunoreactive [106]. Moreover, PGP 9.5- immunoreactive nerve fibers in the skin are more numerous than those revealed by GAP-43 [107]. GAP-43 and PGP 9.5 have been reported to be equally sensitive markers of nerve fibers of the rat autonomic nervous system [8].

***Neuron specific enolase***

Enolase is an enzyme, necessary for anaerobic conversion of glucose to metabolites suitable for oxidation. The brain tissue contains a specific form of enolase that differs structurally, functionally and immunologically from the enolase present in other tissues [108]. Neuron-specific enolase (NSE) is a common marker for both neurons and endocrine cells in the gut [4]. It is also expressed by nonneuronal cells like the parafollicular cells of the thyroid gland, adrenal medullary chromaffin cells, glandular cells of the pituitary and the cells in the islets of Langerhans in the pancreas [108]. Changes in NSE levels have been demonstrated in correlation with neuronal differentiation and in response to injury [6].

***Synaptophysin***

Synaptophysin is a structural component of synaptic and neuroendocrine vesicles and it has been used as a neuronal marker [7].

## ULCERATIVE COLITIS AND ENTERIC NERVOUS SYSTEM

Ulcerative colitis (UC) is an inflammatory disease of colon of unknown etiology [19]. It occurs much more often in industrialized than in third-world countries. Men are slightly more likely to develop ulcerative colitis [109]. Clinical symptoms are rectal bleeding, diarrhea, abdominal pain, and weight loss. The disease is acute or chronic with unpredictable relapses and remissions [110; 111]. Microscopically, UC is primarily a mucosal disease. The traditional histological pathognomonic feature of UC is crypt abscess with accumulation of neutrophilic leukocytes in the lamina propria. Irregular villous mucosal surface, decrease in the mucus content, and distortion or atrophy of crypts support the diagnosis of UC. Severe form of UC includes destruction of glands and ulcerations. These histological changes can be apparent to some degree in UC even with normal appearing finding on endoscopy. Although there is no curative medical treatment, management of the disease remains primarily medical [112]. However, surgery continues to have a major role in management of UC, because it may save the patient's life, eliminate the long-term risk of cancer [113], and abolish the disease. Surgery is indicated in UC either because of failure of medical treatment, or because of acute complications such as fulminant colitis, toxic megacolon, perforation, hemorrhage obstruction, or chronic complications such as risk of cancer, and extraintestinal manifestations [114]. About 31%-45% of UC patients requires colectomy in the long run [115].

In 1953 Storsteen, Kernohan and Bargen reported on 2-3-fold increase in the number of myenteric ganglion cells in UC [20]. The precise mechanism for the hyperplasia remained obscure. Several studies have revealed changes in the neurotransmitter content of the gut, but a clearly defined profile of change has not emerged. Studies focused on sympathetic nerves have described an increase in catecholamine content of adrenergic nerves in UC [21]. Substance P concentration is increased in inflamed colonic mucosa of UC patients [116; 117]. Quantitative histochemical studies by our group and others have indicated that this change is mainly due to increased number of substance P-immunoreactive nerve fibers in the lamina propria [22; 25]. Likewise, the number of substance P receptors is increased in UC colon [82; 118]. Substance P immunoreactivity has also been reported to decrease in severe ulcerative colitis [22; 23]. VIP-immunoreactive nerves have been reported to decrease in ulcerative colitis [23; 24], however some authors have demonstrated no changes in the colonic content of VIP in patients with inflammatory bowel disease [119].

In the rabbit and rat experimental colitis, ablation of sensory nerve fibers by chronic treatment with neurotoxin capsaicin significantly worsened the inflammation in acute colitis and subacute colitis [120; 121]. Moreover, substance P receptor antagonist reduces the severity of colitis and has beneficial effects on the concomitant alterations of contractility [83].

## POUCHITIS

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA), first described by Parks and Nicholls in 1978, has become an established surgical alternative for most patients with ulcerative colitis [122]. In this operation all diseased mucosa is removed and furthermore transanal defecation and continence are preserved. The most frequent long-term complication of IPAA performed on UC patients is inflammation of the ileal reservoir, termed "pouchitis". Pouchitis is a clinical syndrome of deterioration of IPAA function with diarrhea and acute inflammation and/or ulceration of the pouch, as assessed by endoscopy and biopsy histology. In our clinic, the cumulative risk of acute pouchitis and chronic pouchitis was 28% and 5.2%, respectively, at 11 years after surgery [28]. Even higher values of cumulative risk have been reported. In the Mayo clinic the cumulative risk of pouchitis at one, two, five and 10 years after surgery was 15 %, 23 %, 36 %, and 46 % respectively [27].

The similarities in mucosal morphology between pouch ileum and UC colon and the action of the same inflammatory mediators in pouchitis and UC suggest that the pathogenesis of pouchitis and that of UC may be similar [123-126]. This view is supported by the observation that pouchitis rarely develops in a reservoir constructed for reasons other than UC [123]. The other suggested etiologies of pouchitis are stasis, nutritional deficiencies, ischemia or bacterial overgrowth [127].

## NITRIC OXIDE AND ULCERATIVE COLITIS

Increased plasma nitrite levels have been measured in UC patients [128], but the precise etiology of this finding has remained obscure. Increased level of citrulline, the co-product of NO synthesis has been reported in the rectal biopsy of patients with ulcerative colitis [129]. Furthermore, increased level of NO in luminal gas has been measured in the colon in patients with UC [130]. Finally, NOS activity increases eightfold higher in the mucosa of UC colon than in control mucosa [131]. As mentioned above, NOS-2-expression is induced in the epithelial cells of UC colon [26; 132-134]. Contradictory results have been published on association of NOS-2 immunoreactivity with the degree of UC [26; 132; 134]. There is some discrepancy as for NOS-2 immunoreactivity in the cells of lamina propria or submucosa in UC specimens [26; 132; 134]. No reports about NOS-1 and NOS-3 concerning the UC are available.

There is growing evidence that endogenous NO regulates mucosal barrier integrity under physiological conditions and counts for the increase in mucosal permeability associated with acute pathophysiological states [11; 135]. NO may have protective functions in gut epithelium. These include the maintenance of blood flow, inhibition of platelet and leukocyte adhesion and/or aggregation within the vasculature, modulation of mast cell reactivity, and scavenging of reactive oxygen metabolites such as superoxide.

In active UC, excess production of superoxide and hydrogen peroxide is generated by activated leukocytes. These reactive oxidative metabolites and NO can produce peroxynitrite. Peroxynitrate can nitrate tyrosin and produce 3-nitrotyrosine. The immunostaining of 3-nitrotyrosine localized in epithelial cells and in lamina propria [26; 132]. However, a recent study failed to show 3-nitrotyrosine immunoreactivity in epithelial cells, only in some cells in the lamina propria [134].

The dilatation of colon in toxic megacolon syndrome has been attributed to NO [136]. In an animal model of UC, NOS-2-deficient mice develop more severe experimental colitis and recover more slowly than wild type, NOS-2-expressing control animals [137]. NOS inhibitor $N^G$-nitro-L-arginine (L-NNA) aggravates the course of acetic acid-induced colitis in rats [138]. Furthermore, NO-releasing derivative of mesalamine (5-ASA) enhances the anti-inflammatory effect of mesalamine in rats [139]. In contrast, studies using inhibitors of NOS in experimental colitis would suggest that inhibition of NO production will reduce the intestinal inflammation and destruction [140; 141]

## SMALL INTESTINAL ADAPTATION TO RESECTION

Massive small bowel resection results in considerable morphological and functional changes in the remaining intestine [29, 142, 31; 143; 144]. In the rat, mucosal surface and villous height increase, while disaccharidase and peptidase activities decrease. The remaining intestine also dilates, but no significant increase in its length occurs [29; 145; 146]. Similar morphological adaptation has been reported in the dog and the rabbit. In contrast to the rat, resection-induced lengthening of remaining intestine has been reported in the human and the pig [31; 147; 148]. In resected piglets macroscopic adaptation (as measured by ileum length and circumference) appears to be completed in eight weeks after the operation, whereas the increase in the villous height is not observable until 14 weeks postoperatively [31]. This adaptation increases the absorptive capacity of the remaining intestine and aims to maintain nutrient, fluid, and electrolyte balance. The adaptation involves hyperplasia of the mucosa with an increased number of microvilli and a net increase in the absorptive surface area.

Luminal nutrition, humoral factors, and pancreaticobiliary secretions have been proposed as mediators of the adaptation [149; 150]. These may stimulate the synthesis or release of enterotrophic regulatory peptides, such as the enteroglucagon family of peptides (glukagon like peptide-2) and gastrin and growth factors, such as epidermal growth factor and insulin-like growth factor-I [149; 151-153]. Neural factors are also important. Vagotomy in pigs prevents adaptive increase in weight per unit length of residual intestine after resection and sympathectomy in rats decreases the cell proliferation and reduces mucosal mass [151].

Profound motor changes occur in the small intestinal remnant following extensive resection [154-157]. One of these is prolongation of small intestinal transit time, the mechanism of which is poorly understood. Massive small bowel resection directly affects neuropeptide levels in the submucous plexus, resulting in an increased size of the VIP-immunoreactive neurons, while somatostatin -immunoreactive neurons remain unchanged [158]. In contrast, VIP tissue concentration in the mucosa and the muscle layer decrease after massive small bowel resection, and somatostatin content remains constant [159]. Moreover, CGRP content increases, and NPY content remains constant in muscle layer after resection [159]. No quantitative estimation of neuropeptide-containing nerves in resected gut has been reported.

## *AIMS OF THE STUDY*

The aim of this study was to examine how inflammation and adaptation change the expression of neurotransmitters in the enteric nervous system. Particular emphasis was put on the role of substance P and nitric oxide as mediators of neurogenic inflammation.

Specific aims were

1. to develop a quantitative morphometric method for evaluation of neuronal changes in tissue specimens of human gut.

2. to study whether the changes caused by ulcerative colitis in the innervation of colon are specific for substance P;

3. to study correlative changes in expression of neuronal, inducible, and endothelial NOS in ulcerative colitis;

4. to study the correlation between the clinical degree of pouchitis and the expression of inducible and endothelial NOS in ileum reservoir;

5. to study whether the changes observed after experimental short bowel adaptation can be explained by altered innervation.

# *SPECIMENS AND METHODS*

## SPECIMENS

### *Animals (V)*

Ten outbred female domestic pigs weighing between 19-25 kg were used for the experiment. Pigs were chosen as experimental animals because pig gastrointestinal anatomy and feeding habits resemble those of humans.

All the animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals formulated and prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985). The authorization to perform this study was given by the Provincial Government of Uusimaa in accordance to Finnish legislation.

Massive small bowel resection was performed in five 3-month-old pigs (weight 21-25 kg, mean ± SEM 23 ± 0.7 kg). The proximal resection was chosen because the ileum has more adaptive capacity than jejunum [30]. The proximal 75 % of the small bowel was resected from distal to the ligament of Treitz, so that the resected bowel included most of the jejunum and 50 % of the ileum. Bowel continuity was restored by end-to-end jejunoileal anastomoses using a one–layer seromuscular running stitch of 5-0 polyglyconate monofilament. During the operation, a whole-wall specimen was taken from the proximal part of the remaining ileum. The operated pigs and age-matched controls (N=5, weight 19-21 kg, mean ± SEM 19.4 ± 0.5 kg) were raised under standard conditions and allowed to eat ad libitum.

After two months, whole wall specimens of the proximal part of the remaining ileum in both groups were obtained for histological and immunohistochemical studies. We also took specimens of the corresponding ileal segment of 5-month-old unoperated pigs. Since no transsection-induced neuronal changes beyond 10 cm from the anastomosis have been reported [160] and since our specimens were taken 150 mm distally from the anastomosis, no sham operation was considered necessary. This strategy is supported by the following observations. The descending projections of the rat jejunal substance P-, VIP-, somatostatin-, and enkephalin-immunoreactive neurons are shorter than 8 mm, while galanin neurons have descending projections of approximately 20 mm in length [161]. In the dog neuronal projections run for up to about 30mm. The ascending projections are even shorter. In the pig specifically, the enteric projections, with the exception of serotonergic fibers, do not extend for more than a few millimeters (Timmermans, personal communication).

### *Human tissue specimens (I-IV)*

The study was carried out in the Gastrointestinal and Endocrinological Department of Surgery, Helsinki University Central Hospital during 1997-1999.

Oral or written informed consent was obtained from each patient before tissue sampling. The Ethics Committee of the Helsinki University Hospital has approved of the study protocol. (Table 2.).

**Table 2. Specimens**

| Specimens | Number of patients | Study |
|---|---|---|
| Whole wall gut: | | |
| Normal colon | 3 | I |
| | 8 | II |
| | 14 | III |
| Normal ileum | 3 | I |
| UC colon | 10 | II |
| | 13 | III |
| Mucosal biopsies: | | |
| UC ileum | 8 | IV |
| Normal pouch | 15 | IV |
| Chronic asymptomatic pouchitis | 8 | IV |
| Chronic active pouchitis | 11 | IV |
| Acute pouchitis | 11 | IV |

Whole-wall gut specimens

*Normal colon and ileum (I, II, III)*

Specimens of normal colon and ileum were taken from 24 adult patients undergoing resection of colon for treatment of colon or rectum neoplasia at Helsinki University Central Hospital during 1997-1999. The mean age of the patients was 66 years (range 38-83 years, 12 women and 12 men). None of the patients had bowel obstructions or any other colon disease. Whole-wall specimens were taken from the macroscopically normal margin of the resected colon or ileum.

*Ulcerative colitis (II, III)*

Several whole-wall specimens were taken from resected bowel of 19 patients with UC. The mean age of the patients was 37.7 years (range 22-77 years; 10 women and 9 men). The specimens were taken from the least affected region of the colon and from the moderately affected colon. Some specimens were also taken from ulcerated regions with destroyed epithelium (II). In the study II the mean age of the patients was 31 years range 22-46 years; six women and four men. The patients were operated on for failed conservative treatment or for side effects of corticosteroids. One patient was operated on for moderate

dysplasia and primary sclerosing cholangitis. None of the patients were operated on for fulminant colitis. All patients received 5-amino-salicylic acid treatment orally; 8/10 patients received also oral corticosteroids. Besides the corticosteroid therapy, one patient received azathioprine. The mean duration of the disease was 7 years (range 1-15 years). In study III the mean age of the patients was 41 years (range 23-77 years, eight women and six men). The patients were operated on for failed conservative treatment or for side effects of corticosteroids. Eleven out of 14 patients received 5-aminosalicylic acid, 13/14 received oral corticosteroids and one patient received azathioprine. Mean duration of the disease was 10 years (range 1-22 years).

### Mucosal biopsy specimens

Mucosal biopsy specimens were taken from terminal ileum and ileum reservoir of UC patients (IV). Forty patients who had undergone restorative proctocolectomy with construction of ileal reservoir were included in this study. In all patients a two-limbed J-shaped ileal pouch had been constructed. The diagnosis of pouchitis was based on clinical, endoscopic and histological criteria. The main clinical symptom was an increase in defecation frequency with loose to watery stools. Abdominal cramping, bloody stools and systemic malaise were less common. In endoscopy, mucosal edema and erythema, contact bleeding, friability and ulcerations were typical for pouchitis. The histological findings in all specimens of pouchitis showed an increased number of neutrophils in the lamina propria and/or crypt abscesses and superficial erosions and atrophy of villi.

The patients were divided in five groups:
1. To obtain a control, biopsies were taken from normal terminal ileum of UC patients (N=8) during operation before reconstruction of the ileum reservoir. Examination by a pathologist revealed normal ileal histology.

2. No pouchitis (N=15): Most patients were symptomless. If they had abdominal cramps or high frequency defecation, pouchitis was excluded by endoscopy. The endoscopic finding was normal pouch mucosa, and microscopically the finding mostly showed chronic inflammatory cell infiltration in the lamina propria. In some specimens, villus atrophy with colon metaplasia was found. Sometimes slight granulocyte infiltration was present without evidence of endoscopic signs of pouchitis or clinical symptoms of pouchitis. The time of endoscopy varied from 4 to 83 months after pouch reconstruction.

3. Chronic asymptomatic pouchitis (N=8): This group includes patients who had had chronic relapsing inflammation in the pouch mucosa and received medication. At the time of the biopsy, the symptoms were largely decreased. However, the endoscopic finding revealed some small erosions in the mucosa, erythema or edema. In some patients the mucosa appeared quite normal. Upon histological examination, chronic inflammation was found and occasionally infiltration of neutrophils was observed. The time of endoscopy varied from 5 to 83 months after pouch reconstruction.

4. Chronic active pouchitis with exacerbated symptoms (N=11): This group showed the findings of acute pouchitis (see below). The patients had a history of three or more relapsing pouchitis episodes per year, or the symptoms worsened soon after finishing medication. The time of endoscopy varied from 12 to 88 months after pouch reconstruction.

5. Acute pouchitis (N=11): All the patients had symptoms of pouchitis and showed endoscopic and histological findings of acute inflammation. These patients did not have a history of chronic pouchitis. The time of endoscopy varied from 3 to 37 months after pouch reconstruction.

Six biopsies from the each pouch were taken during sigmoidoscopy from the body of the pouch using a standard biopsy forceps. The suture or staple lines and visible ulcerations were avoided.

Salivary gland specimens

Oral surgeon took salivary gland specimens used for comparison for diagnostic purposes. The routine histological staining revealed normal gland morphology.

## METHODS

### *Histology*

Sections of the whole wall specimens (I-III) were cut through formalin-fixed, paraffin–embedded tissue blocks and stained with Herovici-van Gieson method. The criteria of histopathological diagnosis were based on examination by a pathologist.

In study IV, a pathologist unaware of the patient's clinical data examined the mucosal biopsy specimens for histological evaluation.

The presence of active inflammation was recorded and scored semiquantitatively from 0 to 3 (0=absent, 1=mild, 2= moderate, 3= severe). The inflammation was recorded as active when conspicuous mucosal neutrophils or erosions were found. The villus atrophy was recorded and scored from 0 to 3 (0= normal, high villus profiles, 1= low and deformed villus profiles, 2= focal few, deformed villus profiles 3= no villus profiles).

### *Cytochemistry*

Preliminary studies were performed to find out the optimal fixation time and dilution for each staining.

Specimens were immersed in 4% paraformaldehyde in phosphate–buffered saline (PBS), pH 7.2, for 8 hr (I-III), 2 hr (IV) or 24 hr (V) and then transferred into 20 % sucrose in PBS. The specimens were embedded in freezing gel and

10 μm cryostat sections were cut on chrome-alum-gelatin-coated glass slides. Specimens for NOS-3 immunostaining were frozen directly in liquid nitrogen and cryostat sections were immersed in 4 % paraformaldehyde in PBS for 15 min.

For immunohistochemistry the sections were incubated for 20 min with 5 % normal rabbit or swine serum at room temperature, and overnight with the primary antiserum diluted in PBS at +4°C.

Staining detection was based on two different methods: indirect immunofluorescence or avidin-biotin-complex (ABC) method. After the primary incubation, the glasses were rinsed, incubated with secondary antibody for 1 h at room temperature.

In indirect immunofluorescence method secondary antibodies were fluorescein-conjugated sheep anti-mouse IgG (1:300 in PBS, Jackson) for mouse-raised antibodies against GAP-43, synaptophysin, or NOS-1, and fluorescein-conjugated swine anti-rabbit (1:200 in PBS, Dako F205) for rabbit-raised antibodies against VIP-, substance P, enkephalin, galanin, somatostatin, PGP 9.5, NSE, or NOS 1. After fluorescein staining method the preparation was stained with 0.05 % Pontamine Sky Blue for 10 min [162] to diminish background fluorescence (I, III).

If ABC staining method (Vector ABC kit.) was used, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 5 min before the staining process (II, III, IV).

### Primary antibodies

The sources and dilutions of the primary antibodies used are shown in Table 3. The antibodies against GAP-43, PGP 9.5, NSE and synaptophysin were examined as neuronal markers. Neuropeptide antibodies included those against VIP, substance P, somatostatin, enkephalin, galanin, and CGRP. For nitric oxide synthase histochemistry two different antibodies against NOS-1 (neuronal NOS) and one for NOS-2 (inducible NOS) and one for NOS-3 (endothelial NOS) were used.

**Table 3. Primary antibodies**

| Primary antibody | Source | Manufacturer | Dilution | Staining method | Study |
|---|---|---|---|---|---|
| VIP | rabbit | Incstar | 1/500 | IF | I, V |
| VIP | rabbit | Incstar | 1/4000 | ABC | III |
| Substance P | rabbit | Incstar | 1/500 | IF | I, V |
| Substance P | rat | Chemicon | 1/100 | ABC | II |
| Galanin | rabbit | Incstar | 1/500 | IF | I, V |
| Somatostatin | rabbit | Incstar | 1/500 | IF | V |
| Enkephalin | rabbit | Incstar | 1/500 | IF | I, V |
| CGRP | rabbit | Amersham | 1/500 | IF | I, II, V |
| GAP-43 | mouse | Boehringer | 1/500 | IF | I |
| | | | | ABC | II, III |
| PGP 9.5 | rabbit | Affinity | 1/2000 | IF | I |
| NSE | rabbit | Sigma | 1/1000 | IF | I |
| | | | 1/8000 | ABC | II |
| Synaptophysin | rabbit | Sigma | 1/500 | IF | I |
| NOS-1 | mouse | Sigma | 1/2000 | ABC | III |
| NOS-1 | rabbit | Diasorin | 1/8000 | ABC, IF | III |
| NOS-2 | rabbit | Santa Cruz Biotechnology | 1/4000 | ABC | III, IV |
| NOS-3 | mouse | Affinity | 1/4000 | ABC | III, IV |

Incstar, Stillwater, MN, US; Diasorin, Stillwater, MN, US; Chemicon, Temecula, CA, US; Santa Cruz Biotechnology CA, US; Sigma, St. Louis, US; Amersham, Buckinghamshire UK; Affinity, Exeter, UK; Boehringer, Mannheim, Germany.

### Double staining

For the double staining experiments, mouse anti-GAP-43 and rabbit-raised neuropeptide antibodies were incubated simultaneously, and the secondary antibodies consecutively. The second antibody for GAP-43 was fluorescein-conjugated sheep anti-mouse IgG and for neuropeptide-antibodies was rhodamine-conjugated swine anti-rabbit IgG (1:100, DAKO R156) (I).

### Staining specificity

For specificity controls, the primary antiserum was omitted from some sections, which resulted in negative staining. Neuropeptide immunoreactivity totally disappeared after preincubation with 0.1 μM or 1μM solution of the corresponding neuropeptide. The specificity of the following antibodies used in the present study has been characterized elsewhere: anti-substance P, batch 104560 [25] and anti-enkephalin, batch 935 [163], anti-CGRP [164], anti-GAP-43, clone 91E12 [91], anti-NSE, batch AB951 [5], anti-synaptophysin, clone SVP-38 [7]. Based on the documentation provided by the manufacturer, PGP 9.5 antiserum, batch Z00705, recognizes a 24kD band on Western blot

representing the PGP 9.5 peptide in lysates of whole rat brain and human neuroblastoma cell line. NOS-1 immunoreactivity totally disappeared after preincubation of the antiserum with 1μM solution of corresponding NOS-1 peptide (Sigma, Diasorin). NOS-2 immunoreactivity totally disappeared after preincubation of the antiserum with 1 μM solution of NOS-2 peptide (Santa Cruz Biotechnology). For NOS-3 antibodies the corresponding peptide was not available.
Double staining. The possibility of the secondary antibodies cross-reacting with each other was excluded by incubating the specimen first with one of the rabbit-raised antibodies followed by anti-mouse secondary antibody. Correspondingly, specimen stained with mouse-raised antibody was incubated with anti-rabbit secondary antibody. No labeling was observed in either case.

### *Quantitation of immunoreactivity*

Immunoreactivity score

One investigator who was unaware of the clinical and histological details performed the visual estimation of immunostaining of nerve fibers both in terms of the number and intensity. Immunoreactivity was graded from 0 to 3 (0 no reactivity, 1 sparse, 2 moderate, 3 dense/high immunoreactivity) (I, III, IV, V)

Morphometry

The specimens were examined by Leitz Aristoplan epi-illumination fluorescence microscope (I, III, V) or Leica DMLS microscope (II, III, IV) and photographed. The investigator was unaware of the clinical data of the specimen. Three or five sections were randomly selected and photographed (fluorescence) and the photographs were digitized either through a video camera and digitizing board (PC vision Plus, Image Technology) (V) or with Hewlett Packard Scanjet IIcx scanner (I). For some studies, randomly selected sections were digitized from light microscope under standardized circumstances through a video camera connected to the microscope, and digitizing board (II, III). The images were analyzed and stored in the computer using the Sigma Scan Pro 4.0 software (SPSS Science, Erkrath, Germany).

The threshold values for different stainings were determined separately, and the same threshold value was used for all images stained for each particular antibody. After subtracting the background, an intensity histogram of this area was obtained and the threshold of significant intensity was determined by comparing the digitized image with the original view through the microscope or with the corresponding photomicrographs. The pixels representing values below the threshold value were electronically removed.

*Lamina propria.* From sections containing longitudinal villous profiles, the area measured was determined by the epithelial basement membrane of the villus and the muscularis mucosae. In the study V, a value for specific neuropeptide immunoreactivity, referred to as the immunoreactivity index, was obtained by

summing the total number of pixels exceeding the threshold and by dividing this value by the area. This value takes into consideration all structures exhibiting specific immunoreactivity, regardless of their fluorescence intensity, and represents changes in the number of nerve fibers. Moreover, the intensity-based immunoreactivity index was calculated (II, III, V) by multiplying each intensity value by the number of pixels exhibiting this intensity value. This value together with the immunoreactivity index can be used for estimation of changes in the fluorescence intensity of nerve fibers.

*Circular muscle layer*. From the sections containing transverse sections of nerve fibers, three (II, III, V) or five (I) sections were randomly selected, and threshold values were determined as described above. In study V, the immunoreactivity index and the intensity based immunoreactivity index were calculated in the same way as in the lamina propria. To obtain an estimate of nerve fiber density (I, II, III), the number of nerve profiles per unit area and the total area represented by immunoreactive pixels in the measured area were determined. To obtain an estimate of the intensity of immunoreactivity, the total intensity, i.e. the sum of intensity values of all profiles exceeding the background was calculated for standard area of measurements. To obtain an estimate of the thickness of the nerve fibers, the mean perimeter of immunoreactive nerve fiber profiles was measured. For each profile, shape factor SF was calculated using the formula $SF=4\pi\times area/(perimeter)^2$. This parameter indicates how circular a profile is, the value for a circle being 1.0 and that of a line approaching 0. To exclude oblique sections, only those profiles were accepted the SF of which was 0.4 or greater. To exclude nonspecific fluorescent aggregates, profiles the area of which was under 4 pixels were omitted. Since some sections contained occasional profiles, which based on their size or shape were obviously not transected nerve fibers, it was decided to set an upper limit of 300 pixels for the profile area. The values obtained from the three sections of each specimen were averaged.

*Myenteric ganglia*. One out of five sections of each specimen stained for NSE was selected and photographed under standardized conditions so that the whole ganglion chain profile of the section was included to obtain an estimate of ganglion volume. The photographs were digitized with Hewlett Packard Scanjet IIcx scanner. The total (cumulative) area of ganglion profiles in each section was calculated and the value was divided by the length of the ganglion chain. Average intensity of substance P immunoreactivity of randomly selected ganglion profiles was measured for each specimen (II).

### *Statistics*

The results are expressed as the mean $\pm$ the standard error of mean. ANOVA was used for statistical analysis when the possibility was examined whether the variance within one or more groups differs significantly from the variance of the whole material (II, III, IV). The Student's t-test (I, II, III, V) and Wilcoxon signed rank test (I) or Wilcoxon rank sum test with Bonferroni correction and Kruskall-Wallis multiple comparison Z-value test (IV) were used for comparisons among

the groups. Linear -by-linear association test was also used in the study IV. The significance of the correlation between active inflammation score and NOS-2 immunoreactivity score was calculated by non-linear regression analysis (Kendall´s tau estimation). The calculations were made using Sigma Plot for Windows Version 4.0 (SPSS Inc., Germany) or Statexact 4 software (Cytel Inc., US) (IV).

# *RESULTS*

## NEURONAL MARKERS IN MATURE HUMAN INTESTINE (I)

### *GAP-43*

The distribution of GAP-43 was similar in the ileum and colon. In the mucosa GAP-43-immunoreactive nerve fibers were numerous. Nerve fibers extended up to the tips of the villi and formed a dense network at the base of the villus. Muscularis mucosae and the submucous layer also contained a network of individual GAP-43-immunoreactive fibers. The submucous layer also showed thick fiber bundles and ganglia containing a few neurons. In the muscle layer, GAP-43-immunoreactive nerve fibers were abundant and in both the circular and longitudinal layer their course was mostly parallel to that of the muscle fibers. The muscle cell membrane was nonreactive for GAP-43. In the ganglia, a network of GAP-43-immunoreactive fibers surrounded the neurons, particularly densely in the myenteric ganglia. Therefore, it was difficult to evaluate the intensity of the somatic immunoreactivity. However, the impression was that the neuronal somata lacked immunoreactivity (myenteric ganglia) or that the immunoreactivity was weak at the most (submucous ganglia).

### *Co-localization of GAP-43 with neuropeptides*

Double labeling studies were carried out to assess the extent of coexistence of GAP-43 and the neuropeptides VIP, substance P, galanin, and enkephalin. GAP-43 partially co-localized in the nerve fibers with every neuropeptide studied. All VIP-, substance P, galanin and enkephalin-immunoreactive fibers also showed GAP-43 reactivity. However, GAP-43-immunoreactive fibers were found that lacked VIP, substance P, or galanin immunoreactivity. Ganglion cell bodies showing VIP, substance P, galanin, or enkephalin immunoreactivity lacked GAP-43 immunoreactivity.

### *Comparison of synaptophysin, NSE, PGP 9.5 and GAP-43*

Lamina propria contained numerous thin synaptophysin- and NSE-immunoreactive nerve fibers. Many cells in the villous core were weakly or moderately stained by both antibodies. Consecutive sections stained with PGP 9.5 antibody revealed a greater number of reactive nerve fibers. GAP-43 antiserum stained a dense network of nerve fibers but no cells. The number of stained nerve fibers per unit area was even greater than PGP 9.5-immunoreactive fibers, and the fibers appeared more sharply demarcated. The distribution of GAP-43-, PGP 9.5 and NSE staining was similar in the muscularis mucosa. However this layer contained fewer synaptophysin-immunoreactive fibers. All four stainings showed immunoreactive fibers around blood vessels. Likewise, all stainings revealed nerve fibers in the peripheral regions of the Payer's patches.

***Morphometric analysis of GAP-43, PGP 9.5 and NSE immunoreactivities***
In the colon, the mean number of GAP-43-immunoreactive nerve profiles per unit area was 11 % higher than that of PGP 9.5- ($p<0.05$) and 33 % higher than that of NSE-immunoreactive fibers ($p<0.01$) The average number of pixels in a single profile was 52 % greater for GAP-43 than for PGP 9.5 ($p<0.01$) and 60 % greater than for NSE ($p<0.01$). The mean perimeter of the GAP-43-immunoreactive profiles was 24% greater than that of PGP 9.5 -($p<0.01$) and 27 % greater than that of NSE-immunoreactive fibers ($p<0.01$). The mean intensity of pixels in each profile was 6 % greater in the GAP-43-stained sections than in the PGP 9.5- ($p<0.01$) and 8 % greater than in the NSE-($p<0.01$) stained sections. The mean total intensity of the GAP-immunoreactive profiles was 70 % greater than that of NSE-immunoreactive profiles ($p<0.01$) and 60 % greater than that of PGP 9.5-immunoreactive profiles ($p<0.01$). In the colon, there was no significant difference between the examined parameters of PGP 9.5 and NSE stainings.

In the ileum, the mean number of GAP-43-immunoreactive profiles per unit area was 13 % higher than that of PGP 9.5- ($p<0.05$) and 121 % higher than that of NSE- immunoreactive fibers ($p<0.001$). The value for PGP 9.5 was 96 % higher than that of NSE ($p<0.001$). The mean number of pixels in a single profile was 12 % higher for GAP-43 staining than for PGP 9.5 (ns) and 84 % higher than for NSE ($p<0.001$). The value of PGP 9.5 was 64 % higher than that of NSE ($p<0.001$). There was no significant difference in the value for the average intensity of pixels or for the perimeter between GAP-43 and PGP 9.5. For both GAP-43 and PGP 9.5 the values were significantly greater than that of NSE ($p<0.01$). The average total intensity of GAP-43-immunoreactive profiles was 13 % higher than that for PGP 9.5 (ns) and 105 % higher than that for NSE ($p<0.001$). The value for PGP 9.5 was 80% greater than that for NSE ($p<0.001$).

***NSE, PGP 9.5 and GAP-43 immunoreactivities in salivary glands***
For comparison, NSE, PGP 9.5 and GAP-43 immunoreactivities were also examined in the labial and submandibular salivary glands. Nerve fibers around the acini, tubuli, ducts and blood vessels showed immunoreactivity for all three antigens. The density of NSE-stained fibers was clearly lower than that of PGP 9.5- or GAP-43-immunoreactive ones, which revealed a very rich network of fibers throughout the gland. No essential difference was observed between PGP 9.5 and GAP-43 staining.

## CHANGES IN THE MORHOLOGY OF ENTERIC NERVOUS SYSTEM IN UC COLON (II)

The distribution of GAP-43-immunoreactive fibers in the lamina propria of least and moderately affected regions of UC colon was similar to that in the normal colon. However, the very severely affected lesions, which contained only destroyed villous profiles or no villi at all, showed very few fragmented or no

GAP-43-immunoreactive fibers. The staining also revealed numerous GAP-43-immunoreactive fibers in the thickened muscularis mucosae. In the circular muscle layer, the GAP-43 -immunoreactive fibers seemed to be more numerous in moderately affected region of colon than in normal colon or in least affected colon. In the ganglia, no essential differences in GAP-43 immunoreactivity between normal colon and UC colon were observed.

***Neuropeptide-containing nerve fibers in UC colon***

Distribution of substance P, VIP, CGRP, somatostatin, enkephalin and galanin in normal and UC colon specimens was estimated by visual examination. Substance P immunoreactivity was increased in UC colon, while no difference between control and UC colon was observed for the other peptides. Therefore, the following analysis was limited to substance P.

Substance P

In normal colon, some thin varicose substance P-immunoreactive fibers were found in the lamina propria. The fibers extended through the villus. Some fibers in the muscularis mucosae were immunoreactive for substance P. In the submucosa, some thick substance P-immunoreactive fiber bundles, and also some thin substance P-immunoreactive fibers around blood vessels were revealed. Several small submucous ganglia contained substance P-immunoreactive neurons. Both muscle layers contained thin substance P-immunoreactive nerve fibers. The myenteric plexus contained some substance P -immunoreactive neurons and quite many substance P-immunoreactive fibers.

Least affected region of UC colon. On visual estimation, the number of substance P-immunoreactive fibers in the lamina propria was greater in ulcerative colitis than that in normal colon. In the muscularis mucosae, submucosa, muscle layer, and in the submucous and myenteric ganglia, the distribution of substance P immunoreactivity was similar to that of normal colon

Moderately affected region of UC colon. The number of substance P-immunoreactive nerve fibers in the lamina propria was markedly increased, as compared to the two other groups. The intensity of nerve fibers seemed also to be higher. The thickened muscularis mucosae contained many fragmented fibers. Distribution of substance P immunoreactivity in the submucous and muscle layer, and in the submucous and myenteric ganglia was similar to that in the other two groups.

***Quantitative measurements***

GAP-43

In the lamina propria, the total intensity of GAP-43-immunoreactivity was 4.79 ± 0.99 (arbitrary units) in normal colon, 4.64 ± 0.78 in least affected UC colon and 5.26 ± 0.90 in moderately affected UC colon. The total area of GAP-43 immunoreactivity was 64 ± 12 in normal colon, 60 ± 8 in least affected UC colon, and 68 ± 1 in moderately affected UC colon. There were no significant

differences between the groups. In the circular muscle layer there were no significant differences in the measured parameters between normal colon and UC colon (Table 4).

**Table 4. Quantitation of substance P and GAP-43 immunoreactivities**

| Group | Normal colon | Least affected UC | Moderately affected UC | |
|---|---|---|---|---|
| ***Lamina propria*** | | | | |
| *GAP-43* | | | | |
| Total area | 64±12 | 60±8 | 68±1 | ns |
| Total intensity | 4.79±0.78 | 4.64±0.78 | 5.29±0.90 | ns |
| *Substance P* | | | | |
| Total area | 8±2 | 17±5 | 30±4 | § *** |
| Total intensity | 0.55±0.15 | 1.30±0.35 | 2.22±0.28 | § *** |
| ***Circular muscle*** | | | | |
| *GAP-43* | | | | |
| Number of nerve fibers | 102±9 | 99±10 | 133±18 | ns |
| Total area | 164.5±34.7 | 149±25.1 | 182.7±30.1 | ns |
| Total intensity | 2691±592 | 2428±428 | 2945±503 | ns |
| Perimeter of a nerve fiber | 14.4±1.3 | 14.3±1.2 | 13.6±0.9 | ns |
| *Substance P* | | | | |
| Number of nerve fibers | 17±2 | 15±3 | 25±6 | ns |
| Total area | 9.68±1.53 | 9.16±2.98 | 11.85±2.77 | ns |
| Total intensity | 134±22 | 128±44 | 157±38 | ns |
| Perimeter of a nerve fiber | 8.1±0.6 | 8.1±0.5 | 7.3±0.5 | ns |

Mean ± SEM, Student's t-test. § $p<0.05$ comparison with least affected and moderately affected UC colon, *** $p<0.001$ comparison with normal colon and moderately affected UC colon.

Substance P

In the lamina propria, total intensity of substance P-immunoreactive nerve fibers was 0.55 ± 0.15 in the normal colon, 1.30 ± 0.35 in least affected UC colon ($p=0.087$) and 2.22 ± 0.28 in moderately affected UC colon ($p<0.001$). The value for least affected UC colon and that for moderately affected UC colon are 136 % and 304 % higher than the control value. The value for moderately affected UC colon is 71 % higher than that for least affected UC colon. Total area of substance P immunoreactive nerve fibers was 8 ± 2 in the normal colon, 17 ± 5 in least affected UC colon ($p=0.12$), and 30 ± 4 in moderately affected UC colon ($p<0.001$). The difference between least affected and moderately affected UC colon was significant ($p<0.05$). The area and total

intensity of substance P immunoreactivity correlated with the degree of UC (Spearman´s correlation test $p<0.001$). Comparison of several parameters in the circular muscle layer revealed no significant differences for substance P immunoreactivity between normal colon and UC colon (Table 4).

Myenteric ganglia

The average intensity of substance P immunoreactivity in myenteric ganglia was 75.8 ± 1.5 in normal colon, 78.1 ± 1.2 in least affected colon, and 77.1 ± 1.2 in moderately affected colon. The differences between the groups are nonsignificant. The average cumulative area of NSE-immunoreactive profile of the myenteric ganglia per unit length of the plexus was 1.39 ± 0.21 in normal colon, 1.86 ± 0.17 in least affected colon, and 1.88 ± 0.15 in moderately affected colon. The differences between the groups were nonsignificant.

## NITRIC OXIDE SYNTHASES IN NORMAL HUMAN COLON AND UC COLON (III)

### *NOS-1 immunoreactivity*

The epithelium and most of lamina propria lacked NOS-1 immunoreactivity. Muscularis mucosae contained a loose plexus of thin, varicose NOS-1-immunoreactive fibers, which occasionally extended a short distance into the lamina propria. Numerous small ganglia in the superficial part of submucosa formed the submucous plexus. It contained a loose NOS-1-immunoreactive fiber plexus, which was continuous with that of the muscularis mucosae. The submucous ganglion neurons were surrounded by NOS-1-immunoreactive terminals but the neurons themselves were nonreactive for NOS-1 with the exception of solitary cells sporadically found in few specimens. Conspicuously, no convincing observation on NOS-1-immunoreactive perivascular innervation was made. The deeper layer of submucosa was devoid of NOS-1-immunoreactive fiber network, although it showed some thicker NOS-1-immunoreactive fiber bundles. The inner, circular muscle layer contained rich NOS-1-immunoreactive innervation, which was mostly visualized as cross-sections of nerve fibers. Occasionally, solitary NOS-1-immunoreactive neurons were observed among the muscle fibers. The outer, longitudinal muscle layer showed only few NOS-1-immunoreactive fibers, which showed as solitary, longitudinal fiber profiles. The myenteric plexus contained bundles of NOS-1-immunoreactive fibers and basket-like NOS-1-immunoreactive terminal networks densely covered the ganglion neurons. Most myenteric ganglion neurons were nonreactive. The number of NOS-1-immunoreactive neurons per ganglion profile varied greatly: some contained none, in some a maximum of about one third of the neurons were reactive. An overall estimate was obtained that approximately 10 % of the myenteric ganglion neurons showed NOS-1 immunoreactivity.

As in controls, lamina propria lacked NOS-1 immunoreactivity in UC colon. In muscularis mucosae of least affected UC specimens, many areas lacked NOS-

1 immunoreactive nerve fibers completely, although in some places few solitary NOS-1 immunoreactive fibers were observed. In moderately affected UC, the reduction was even more conspicuous. NOS-1 staining in the submucosa was indistinguishable from the controls. Likewise, no difference from the control was apparent in NOS-1 immunoreactivity of the muscle layers. The visual impression was confirmed by quantitative morphometric analysis, which revealed no significant differences in the total area or intensity of immunoreactive fiber profiles, fiber thickness or the number of fibers per unit area.

Since NOS and VIP immunoreactivities colocalize in at least some enteric neurons and nerve fibers, it was of interest to study whether the reduction in the number of NOS-1-immunoreactive fibers in the muscularis mucosae correlated with a similar change in VIP-immunoreactivity. However, no obvious difference in the density of VIP-immunoreactive nerve fibers in the muscularis mucosae between control and UC specimens was observed.

***NOS-2 immunoreactivity***

Half the specimens of normal colon completely lacked NOS-2 immunoreactivity, and the other half contained vague epithelial NOS-2 immunoreactivity deep in some crypts. Some least affected UC specimens were completely nonreactive for NOS-2, while in others, local NOS-2-immunoreactivity was observed in some villus tips and in some crypts. In moderately affected UC, uniform epithelial NOS-2 immunoreactivity was consistently seen in the surface epithelium and in the crypts. The reactivity was intense in the apical region of epithelium, while only weak reactivity was seen in the rest of the epithelium. Occasionally cells exhibiting varying NOS-2 staining intensity were seen in the mucosa and submucosa. However, some stained cells were also seen in specimens incubated with antiserum preabsorbed with NOS-2 peptide. Although the possibility cannot be excluded that some specifically NOS-2-reactive cells may be present, their number is very low at any rate.

***NOS-3 immunoreactivity***

In normal colon, NOS-3 antiserum stained exclusively vascular profiles, the gut epithelium being nonreactive. Blood vessels in the lamina propria showed uniformly strong NOS-3 immunoreactivity and they extended throughout the villous core. The submucosa also contained numerous NOS-3-stained blood vessel profiles of greatly varying size. Staining intensity in most vessels was weak to moderate, while some intensely stained profiles were also present. The difference in vascular staining intensity between the lamina propria and submucosa was consistent in all specimens. In the muscle layers, the number of vascular profiles per unit area, as estimated visually, was clearly lower than that in the submucosa, there being no essential difference between the inner and outer muscle layers. Submucous and myenteric ganglion neurons were nonreactive.

In specimens of least affected region of UC, the villi were often thickened. However, the distribution and intensity of NOS-3 immunoreactivity and the density of vascular profiles in lamina propria as well as in the other layers were essentially similar to those of normal colon. In 4 out of 5 specimens the difference in staining intensity between lamina propria and submucosa was clearly observed. In moderately affected UC, marked villus atrophy was evident and the number of NOS-3-immunoreactive profiles was in many areas increased. In 4 out of 5 specimens, the vascular staining intensity in the lamina propria was lower than that in control and in 3 out of 5 specimens no difference in staining intensity between lamina propria and submucosa of the same specimen could be seen. NOS-3 immunoreactivity in the muscle layers was similar to that in controls. As in controls, the submucous and myenteric ganglion neurons were nonreactive.

## NITRIC OXIDE SYNTHASES IN TERMINAL ILEUM AND IN CONTINENT ILEUM RESERVOIR OF UC PATIENTS (IV)

### *NOS-1 immunoreactivity*

Biopsies taken revealed only mucosal structure limited to the upper portion of muscularis mucosae. No NOS-1-immunoreactivity fibers were found in the lamina propria. Thus, no ganglia were included in the biopsy specimens.

### *NOS-2 immunoreactivity*

In 6 out of 8 specimens of normal terminal ileum, no NOS-2 immunoreactivity was observed. Two specimens showed an occasional villous profile with some reactivity in the villus epithelium. In no-pouchitis group, several specimens displayed no NOS-2 reactivity at all, while in others some villi or crypts with NOS-2 immunoreactive epithelium were seen. In asymptomatic chronic pouchitis group, some specimens showed no NOS-2 immunoreactivity, while in most specimens areas of NOS-2 immunoreactive epithelium were consistently observed. In active chronic pouchitis group, only one specimen lacked NOS-2 immunoreactivity, all others regularly showed moderate to extensive epithelial NOS-2 staining. Acute pouchitis group showed NOS-2 staining pattern similar to that of active chronic pouchitis, there being unexceptionally moderate or abundant epithelial NOS-2 staining.

NOS-2 immunoreactivity was almost exclusively confined to the epithelium. With respect to a single epithelial cell, the staining was localized in the apical cytoplasm, while the basal cytoplasm showed only vague reactivity. Only few solitary NOS-2 immunoreactive cells were observed in the lamina propria or submucosa in groups with pouch. No immunoreactive cells were found in the terminal ileum group. No staining was observed in the nerve fibers or in the endothelium in any group.

Semiquantitative analysis

The value of NOS-2 immunoreactivity was 0.25 ± 0.16 in normal ileum of UC patients, 0.67 ± 0.19 in no pouchitis group, 1.19 ± 0.40 in chronic asymptomatic pouchitis group, 2.0 ± 0.23 in chronic active, and 2.18 ± 0.12 in acute pouchitis group.

The values of no-pouchitis group and chronic asymptomatic pouchitis did not differ significantly from normal ileum. The values of both normal ileum and no pouchitis group differed significantly from the values of chronic active and acute pouchitis groups ($p<0.0005$).

***NOS-3 immunoreactivity***

NOS-3 immunoreactivity was confined to the vascular endothelium and no staining was seen in the villus or gland epithelium in any groups. In terminal ileum specimens, as well as in no-pouchitis specimens, NOS-3-immunoreactive vascular profiles were readily observed in the lamina propria and at the base of the villi. However, NOS-3-reactive profiles were only rarely observed in the villus core. By visual examination, chronic asymptomatic, chronic active and acute pouchitis groups showed a greater density of vascular profiles in the lamina propria than the no-pouchitis group. In the pouchitis groups, NOS-3 staining revealed immunoreactive vessels throughout the villus core. No essential difference in the intensity of immunoreaction was observed between the groups.

## ACUTE INFLAMMATION AND VILLUS ATROPHY SCORES (IV)

Acute inflammation score in normal ileum was consistently 0, in no pouchitis-group, 0.53 ± 0.17, in chronic asymptomatic pouchitis group 1.00 ± 0.33, in chronic active pouchitis group 1.80 ± 0.20 and in acute pouchitis 1.64 ± 0.15. The values for all three pouchitis groups differed significantly from that of the normal ileum ($p<0.05$, $p<0.0005$, and $p<0.0005$, respectively). The value for no pouchitis group did not differ from that of the chronic asymptomatic pouchitis group, but did differ from those of chronic active and acute pouchitis groups ($p<0.0005$ and $p<0.001$, respectively).

Both NOS-2 immunoreactivity score, acute inflammation score and villus atrophy score showed a significant correlation to the clinical degree of pouchitis (Linear-by-linear association test, $p<0.01$, $p<0.0001$ and $p<0.0005$, respectively).

NOS-2 immunoreactivity score significantly correlated with the acute inflammation score ($p < 0.0001$, Kendall´s tau estimate 0.654, 95% confidence interval 0.521, 0.790).

In all pouchitis groups marked villus atrophy was found and the scores significantly differed from those of both normal ileum ($p<0.01$, $p<0.01$ and

p<0.005 respectively) and no-pouchitis group (p<0.05, p<0.05 and p<0.005 respectively).

## EFFECTS OF MASSIVE PROXIMAL SMALL BOWEL RESECTION IN THE PIG (V)

Two months after the resection the 5-month-old pigs in the resection group weighed 36-54 kg (46 ± 3.1 kg), and those in the unoperated group 70-74 kg (72 ± 0.7 kg) (p<0.01). The increase in weight was 23 ± 2.7 kg in the resected group and 52 ± 0.7 kg in unoperated group (p<0.001).

### *VIP*

In 3-month-old control pigs numerous VIP-containing nerve fibers were observed in the lamina propria. They extended throughout the core of the villus. Moderate numbers of fibers were seen in the muscularis mucosae, submucosa, and smooth muscle. VIP-immunoreactive cell bodies were found in submucosal and myenteric plexuses. In 5-month-old unoperated pigs the staining of VIP fibers was similar to that in the 3-month-old pigs. In 5-month-old operated pigs VIP-immunoreactive fibers were not as numerous as in 3-or 5-month-old unoperated pigs. In the circular muscle layer, the VIP immunoreactivity index was 1.989 ± 0.108 in the 3-month-old pigs, 1.730 ± 0.115 in the 5-month-old unoperated pigs and 0.0882 ± 0.288 in 5-month-old operated pigs. The change was significant in relation to both unoperated control groups (p< 0.01). In the villus, the VIP immunoreactivity index was 4.372 ± 0.605 in 3-month-old pigs, 3.929 ± 0.407 in 5-month-old pigs and 2.535 ± 0.264 in 5-month-old operated pigs. When the 5-month-old operated piglets were compared to 5-month-old unoperated piglets or to 3-month-old unoperated piglets, the difference in the immunoreactivity of villi was significant (p<0.05). Changes in the intensity- based immunoreactivity of the circular muscle layer and villi were similar to those in the immunoreactivity index.

### *Galanin*

The distribution of galanin immunoreactivity was similar to that of VIP. The number of galanin immunoreactive fibers was not as great as that of VIP-immunoreactive fibers. As was the case for VIP, fewer galanin-immunoreactive fibers were seen in the specimens of 5-month-old operated pigs than in the controls. The galanin immunoreactivity index of circular muscle layer was 1.204 ± 0.199 in the 3-month-old pigs, 1.402 ± 0.137 in the 5-month-old pigs and 0.423 ± 0.238 in the 5-month-old operated pigs. There was no difference between 3- and 5-month-old unoperated pigs, but the operation caused a marked reduction of the galanin immunoreactivity index (p< 0.05). In the immunoreactivity of the villi, we could not measure any significant differences between the groups. Changes in the intensity-based immunoreactivity were similar to those in the immunoreactivity index.

***Enkephalin***

In 3-month-old pigs, many enkephalin-immunoreactive fibers were seen in the circular muscle and in the myenteric plexus. The mucosa, submucosa and longitudinal muscle did not contain any fibers, nor could we see any reactive cell bodies. In the 5-month-old pigs, both unoperated and operated, the distribution of enkephalin-immunoreactive fibers was similar to that in 3-month-old pigs. The enkephalin immunoreactivity index was $0.632 \pm 0.107$ in 3-month-old pigs, $1.306 \pm 0.215$ in 5-month-old control pigs ($p<0.05$ as tested against 3-month-old controls), and $0.449 \pm 0.106$ in the 5-month-old operated pigs ($p<0.05$ as tested against both 5-month-old and 3-month-old control pigs). Changes in the intensity-based immunoreactivity were similar to those in the immunoreactivity index.

***Substance P***

Substance P containing nerve fibers were detected in all layers of the intestine and also in the ganglia. There were a few fibers in the lamina propria, submucosa and in the longitudinal muscle. The number of fibers was moderate in the circular muscle and the myenteric plexus. Visually we observed no difference between these three groups. Therefore no quantitation was attempted.

***Somatostatin***

We observed somatostatin-containing nerve fibers in both the submucous and myenteric plexuses. Both submucosal and myenteric ganglia contained somatostatin-immunoreactive cell bodies. No differences were observed between the three groups.

# *DISCUSSION*

## GAP-43 IS A SUPERIOR NEURONAL MARKER IN HUMAN MATURE INTESTINE

GAP-43 is known as a neuronal membrane-bound protein associated with development and regeneration. Moreover, it is continuously expressed in certain regions of the mature brain [165; 166], as well as in the autonomic nervous system [8]. It has remained unknown whether GAP-43 immunoreactivity correlates with particular neurotransmitters or neuropeptides. The present study demonstrates abundant GAP-43-immuoreactive nerve fibers in the normal human small and large intestine. In previous studies, GAP-43 immunoreactivity has been described in the rat stomach, and small and large intestine [8; 88; 98; 99]. GAP-43-immunoreactive nerves have also been reported in the human small intestine and rectum [9; 69; 100]. All these studies unanimously report on a dense network of GAP-43-immunoreactive nerve fibers in all gut layers. In the present study, the distribution of GAP-43-immunoreactive fibers in the normal human colon was similar to that in the small intestine. Colocalization studies showed that all VIP-, substance P-, galanin- and enkephalin-containing nerve fibers also showed GAP-43 immunoreactivity. Previously VIP has been found to colocalize with GAP-43 in the ferret ileum [9]. In the rat jejunal villi, all electron microscopically identifiable nerve profiles were found to be GAP-43 positive [88]. *Taken together, these observations suggest that GAP-43 is a universal neuronal marker in the mature enteric nervous system.*

Submucous ganglion neurons lacked GAP-43 immunoreactivity and myenteric neurons displayed only very weak GAP-43 immunoreactivity. Stewart and coworkers (1992)[8] found no GAP-43 immunoreactivity in the rat enteric ganglia. Sharkey and coworkers (1990)[9] found weak immunoreaction in the ganglion cells in the rat myenteric plexus and also in the neurons in the submucous plexus in the human duodenum. They also demonstrated by in situ hybridization technique that enteric neurons express GAP-43-mRNA. These results indicate that mature enteric neurons synthesize GAP-43, albeit at low levels.

## COMPARISON OF GAP-43 WITH PGP 9.5 AND NSE

GAP-43 as a neuronal marker has not been compared in the gut with other established neuronal markers, such as PGP 9.5 or NSE. PGP 9.5 is a cytoplasmic protein localized both in the central and peripheral neurons and nerves [5; 101-103]. It has been claimed to be a sensitive immunohistochemical neuronal marker, even better than NSE, for the enteric nervous system in whole mount preparations of mammalian intestine [104; 105]. In the present study visual investigation indicated that GAP-43 reveals a slightly denser nerve fiber

network than PGP 9.5 both in lamina propria and in the muscle layer. The GAP-43-immunoreactive fiber profiles showed greater intensity were thicker and sharper demarcated than those stained with PGP 9.5 antiserum. Better visibility of GAP-43-immunoreactive nerves is likely due not only to greater immunofluorescence intensity per se, but also to different intracellular distribution of the two proteins, GAP-43 being membrane-bound while PGP 9.5 is soluble. An additional factor contributing to the appearance of GAP-43-immunoreactive fibers is immunoreactivity of satellite cells or non-myelinating Schwann cells around enteric nerve fibers. This possibility is based on the observation both in vitro and in vivo that rat glial cells and non-myelinating Schwann cells express GAP-43 [167].

Comparison of GAP-43 and PGP 9.5 has shown that only a fraction of nerve fibers in normal adult pancreas exhibits GAP-43 immunoreactivity, while most if not all nerve fibers are PGP 9.5-immunoreactive [168]. Likewise, PGP 9.5-immunoreactive nerve fibers in the skin are more numerous than those revealed by GAP-43 [107]. This is in agreement with the original concept that GAP-43 is associated with developing or regenerating nerves. However, it contrasts with our observation on the human salivary glands in which GAP-43 immunostaining revealed a somewhat denser plexus of nerve fibers than PGP 9.5, as well as with those reported on the rat autonomic nervous system in which GAP-43 and PGP 9.5 are equally sensitive markers of nerve fibers [8].

NSE is a widely used neuronal marker, although several neuroendocrine and non-neuronal cells also express it [108]. Our observation was similar to that reported previously [4] in showing a NSE-immunoreactive fiber network throughout the lamina propria and muscle layer. However, NSE-immunoreactive profiles were thinner and less numerous than those stained with GAP-43 or PGP 9.5. Nonspecific staining of connective tissue cells in the lamina propria further hindered the visibility of NSE-immunoreactive nerve fibers. No nonspecific staining was seen in the muscle layer. Nevertheless, fewer and less intensively stained nerve fibers were revealed with NSE staining as compared with GAP-43 or PGP 9.5 stainings.

In contrast to nerve fiber staining, NSE was a superior marker of ganglion neurons. All ganglion neurons showed a strong cytoplasmic immunoreactivity. Somatic PGP 9.5 immunoreactivity varied in intensity and GAP-43 revealed only vaguely reactive neuronal somata at the most. Poor staining by GAP-43 antibody is likely due to rapid transportation of the protein into the axons to be incorporated into the axolemma.

Quantitative analysis of the nerve fibers in the circular muscle layer verified our visual observations as for the order of sensitivity of the three markers: GAP-43>PGP 9.5>NSE. Both in the colon and ileum, GAP-43 revealed over 10 % more nerve fibers per unit area than the other two markers, indicating that GAP-43 staining reveals fibers unstained by PGP 9.5 antiserum. The difference in average number of pixels per single profile, a parameter correlating with profile size, was significant in the colon, and the trend was

similar in the ileum. Similar result was obtained when average profile perimeters were compared. These results suggest that the fibers revealed by GAP-43 staining are thicker than those stained by the PGP 9.5 or NSE antibodies. Total intensity, as defined in the present study, sums the intensity values of each pixel in a profile, and is thus sensitive to changes in profile size and/or intensity. Therefore, the difference in total intensity of a single profile between GAP-43-immunoreactive and the other two markers is expected and can be explained by the differences in the number of pixels per profile. In fact, the values for average intensity of profiles were essentially similar for each antibody, the difference between GAP-43 and the other two being only about 1/10 of the difference in total intensity values. *In summary, our results suggest that GAP-43 immunoreactivity reveals more numerous and thicker nerve fibers than PGP 9.5 or NSE immunoreactivities although the true staining intensity is essentially similar for the three markers. In contrast, NSE is a superior marker for neuronal somata.*

## INCREASE IN NERVE DENSITY IN UC INVOLVES EXCLUSIVELY SUBSTANCE P CONTAINING NERVE TERMINALS

This study confirmed the previous results that the density of substance P-immunoreactive fibers in colonic mucosa increases in UC [22; 25]. The increase in substance P immunoreactivity was proportional to the degree of inflammation, when control specimens and least and moderately affected UC specimens were compared. In consecutive sections stained for GAP-43, no corresponding differences were observed. Since GAP-43 is a general neuronal marker in the human gut, this comparison suggests that the increase in nerve density is specific for substance P-containing nerve fibers. This conclusion is in accordance with published literature on neuropeptide changes in UC, lacking reports on increased density for any other peptide than substance P [22-25]. In preliminary studies, we screened the neuropeptides VIP, galanin, somatostatin and CGRP, without observing any increase in their innervation pattern. The density of GAP-43-immunoreactive nerve fibers remaining unchanged in control and UC specimens supports the conclusion that the increase in substance P fibers is not explained by tissue shrinkage caused by UC.

The density of substance P-immunoreactive nerve fibers decreases in severe UC [22; 23]. Similar change occurs in VIP-immunoreactive nerves [23; 24]. This is in line with the present observation on lack of substance P-immunoreactive nerves in severely affected UC specimens. Lack of substance P-containing nerves correlated with subtotal disappearance of GAP-43-immunoreactive nerves. *Taken together, these findings suggest that disappearance or strong decrease in neuropeptide-containing nerves in severe UC is simply due to complete destruction of innervation at the extreme stage of the disease.*

The density of nerve fibers in the lamina propria remained unchanged in mild and moderate degree of UC, as illustrated by constant GAP-43 immunoreactivity. This confirmed that the nerve fibers of enteric nervous

system do not proliferate in mild and moderate UC. *Thus, the increase in substance P immunoreactivity in the lamina propria seems to be caused by increased synthesis of substance P, so that more substance P-immunoreactive fibers become visible.*

Several parameters describing the innervation of the circular muscle layer, such as the density and thickness of substance P-immunoreactive nerves, or intensity of the immunoreaction remained unchanged in mild, moderate or even severe UC as compared to controls. The number of substance P-containing nerves of the circular muscle is not affected by extrinsic denervation [161; 169-171], indicating that these nerves are exclusively derived from gut ganglia. Our finding thus suggests that no marked overall changes in the intrinsic nervous system are induced by UC. This conclusion is substantiated by lack of changes in specimens stained for GAP-43, which reveals the entire nerve fiber population. Thus, UC does not seem to induce significant quantitative changes in muscular innervation. This contrasts with previous findings on thickened nerve fibers in the muscle layer of Crohn´s disease patients [119; 172] suggesting some differences in the pathogenesis of these two states of inflammatory bowel disease.

To examine whether UC is associated with increased size of myenteric plexus ganglia, we measured the cumulative area of ganglion profiles per unit length in sections stained for NSE. The need for these measurements was suggested by previous studies reporting on 2-3-fold increase in the number of myenteric ganglion cells both in Crohn´s disease [172] and UC [20]. In our study, no evidence for ganglion hypertrophy was obtained. The reported tendency for greater values of neuron numbers in UC specimens may be due to shrinkage known to occur in UC colon.

## SUBSTANCE P AND NEUROGENIC INFLAMMATION IN ULCERATIVE COLITIS

Substance P-immunoreactive fibers in the enteric nervous system originate from both the intrinsic (gut ganglia) and extrinsic neurons (primary sensory neurons in the dorsal root ganglia). The intrinsic neurons are quantitatively the most important source of the substance P-containing nerves projecting to mucosal and submucosal blood vessels, mucosal epithelium, the muscle layers, and they also provide interganglionic connections. The extrinsic neurons connect to the gut via sympathetic (splanchnic and hypogastric) and sacral parasympathetic (pelvic) nerves and project to the submucosal and mucosal blood vessels [56]. The present observations, although confirming the finding that the density of substance P-containing nerves is raised by 100-300 % in UC, raise the question which component is responsible for the increased number of substance P fibers.

In Hirschsprung disease the agangliotic segment lacks substance P-immunoreactive nerves in the muscle layer and no neurons are found in the

submucous and myenteric plexi. In contrast, nerves in the lamina propria show normal density. Thus, the conclusion can been made that most of the substance P nerves in the mucosa are extrinsic [173]. Our results do not favor the conclusion that the increase in substance P occurs in intrinsic fibers. Namely, no increase in the intensity of substance P immunoreactivity in the submucous or myenteric ganglia was observed, neither was the density of substance P nerves in the circular muscle, another target of the intrinsic neurons, affected by UC. The possibility remains that these fibers are extrinsic sensory fibers, since also other inflammatory processes, such as psoriasis, arthritis and asthma involve a similar increase in substance P immunoreactivity of sensory nerves [79-81]. Unfortunately, we could not address this question directly, since the neurochemical identification of sensory nerves, i.e. colocalization of substance P and CGRP, does not hold for human gut [174; 175]. *We interpret that the increased density of substance P fibers is due to increased peptide synthesis, and consequently increased visibility of the fibers. Sprouting caused by UC cannot be excluded, although is unlikely, since it should show as an increased immunoreactivity of GAP-43* [92].

Sensory nerve fibers are known to secrete substance P from their peripheral process [76]. Our observation provides a morphological substantiation for the hypothesis that substance P has an important role in ulcerative colitis. This hypothesis has gained extensive support from the following observations: A recent experimental study in mice has shown that neutral endopeptidase, a cell surface enzyme that degrades substance P in the extracellular fluid, terminates dinitrobenzene sulfonic acid-induced colitis in wild type mice [84]. In the neutral endopeptidase knockout mice substance P concentrations were higher and inflammation worse than in wild type mice. Recombinant neutral endopeptidase and substance P receptor antagonist prevented the exacerbated inflammation in knockout mice. Furthermore, the expression of NK1 binding sites by blood vessels and lymphoid aggregates is increased in inflammatory bowel disease [82; 118]. Substance P has been found to enhance mast cell-mediated secretion in active UC, but not in normal mucosa [176]. Mast cell hyperplasia in the gut is a well-known feature of inflammatory bowel disease [177]. Furthermore, increased histamine release has been found in UC [178]. Topical treatment with lidocaine gel in patients with ulcerative proctitis for 3-8 weeks caused a rapid decrease of subjective and objective symptoms. [179]. The present study strengthens the theoretical basis to suggest the use of substance P antagonists in treatment of UC.

## ULCERATIVE COLITIS INDUCES SELECTIVE REDUCTION OF NEURONAL NOS

### *NOS-1 in the normal colon*

Our results on the distribution of NOS-1 in the normal human colon are in line with the previous reports on human colon [14; 15]. We extended these findings by the observations that the submucous ganglion neurons lacked NOS-1 immunoreactivity, while a proportion varying from 0 to 30 % of the cells in each

myenteric ganglion profile were NOS-1-reactive. Based on these findings it can be estimated that intrinsic NOergic innervation of the human colon is derived from a population of myenteric neurons comprising about 10-20 % of all myenteric neurons.

NOS-1-immunoreactive nerve fibers formed a dense innervation pattern in the inner, circular muscle layer and the muscularis mucosae and somewhat less densely innervated the outer, longitudinal muscle layer. The lamina propria was devoid of NOS-1-immunoreactive fibers and the submucosa only rarely showed nerve terminals. The fiber bundles seen in the submucosa are probably formed by the axons traversing the submucosa on their way to muscularis mucosae. Notably, no perivascular NOS-immunoreactive innervation was observed. By contrast, staining with GAP-43 antibody showed abundant nerve terminals in the lamina propria and in the submucosa, including those around the blood vessels. *Thus, our findings suggest that the NOS-containing neurons in human colon primarily contribute to the innervation of intestinal smooth muscle, but do not seem to have any direct role in the regulation of secretion or blood flow.* In experimental animals, NO produced by NOS-1 mediates muscle relaxation through inhibitory junction potential [16; 17; 43]. In the human colon, the role of NOS-1 is less clear: inhibition of NOS reduces non-adrenergic, non-cholinergic relaxation, although the inhibitory junction potential is not affected [18; 85; 180].

***NOS-1 in ulcerative colitis***

Disappearance or marked reduction in the number of NOS-1-immunoreactive nerve fibers in the muscularis mucosae was found in UC specimens. In contrast, no corresponding change was seen in GAP-43- or VIP-stained sections. Moreover, no change in GAP-43 staining pattern was observed in the circular or longitudinal muscle layers. These results demonstrate that UC affects the NOergic innervation specifically in the muscularis mucosae. Our results are supported by a recent report on reduced number of NOS-containing neurons in colonic wall in experimental rat colitis [181]. Two separate mechanisms can be envisaged behind this change: NOS-1-containing nerve fibers may truly disappear or their NOS-1 expression is specifically down regulated in UC. The latter alternative is favored by the observation that no change was observed in the innervation of muscularis mucosae, either as visualized by VIP, a neuropeptide largely colocalizing with NOS [15] or GAP-43, a general neuronal marker. Reduction of muscularis mucosae NOS-1 content in UC may be accompanied with reduced sensitivity to arachidonic acid metabolites and/or reduced excitation-contraction coupling [182], although the causal relationship of these phenomena remains to be elucidated.

## INDUCTION OF NOS-2 IN ULCERATIVE COLITIS

NOS-2 is the inducible NOS isoform, which has been localized, in activated macrophages, neutrophils and several other cell types [36]. Previous studies report that normal human colon lacks NOS-2 [26; 132-134]. Our study by and large

confirms this conclusion, although we regularly observed slight NOS-2 immunoreactivity in the epithelium of some crypts in about half the specimens of normal colon. The difference between the control and UC specimens was marked, and the appearance of NOS-2 immunoreactivity in the epithelium clearly correlated with the degree of the disease. Previously, contradictory results have been published, some studies showing association of NOS-2 immunoreactivity with the degree of UC [132; 134], others reporting lack of such association [26]. Lack of NOS-2 immunoreactivity in normal colon or in mild UC in the previous studies is likely due to differences in the sensitivity of the immunohistochemical methodology. There is some discrepancy as for NOS-2 expression in the cells of lamina propria or submucosa in UC specimens. We, like Dijkstra and coworkers, [134] did not observe but few NOS-2-immunoreactive cells, while others report such reactivity in a minority of inflammatory cells [26] or even in several different cells types [132; 133; 183; 184]. Varying medication of the patients or differences in the specificity of the technique may be responsible for these differences. Several studies have failed to show rapid expression of nitric oxide synthase or production of NO in human macrophages [185].

Expression of NOS-2 in the colon epithelium, particularly in the apical region, in UC is compatible with some protective function of NOS in the injured epithelium. This idea is supported, on one hand, by development of exacerbated UC in NOS-2 deficient mice [137] or in mice treated with NOS-2 inhibitor [138] and, on the other hand, enhanced anti-inflammatory effect of NO-releasing derivative of 5-ASA [139]. Induction of NOS-2 in the apical epithelium may also be a cause for inhibited bacterial invasion in UC [186]. However excess of NO can react with superoxide and produce peroxynitrite. Studies using inhibitors of NOS in experimental colitis would suggest that inhibition of NO production will reduce the intestinal inflammation and destruction [140; 141] NOS-2 may be responsible for increased permeability observed in UC [187], since in experimental animals NO donor induced hyperpermeability of cultured epithelial monolayers [188] and inhibition of NOS-2 prevents endotoxin-induced increase in gut permeability [189].

## REGIONAL DIFFERENCES IN ENDOTHELIAL NOS DISAPPEAR IN ULCERATIVE COLITIS

As expected, NOS-3 was localized exclusively in the vascular endothelium. Surprisingly, the staining intensity of lamina propria vessels in the normal colon was consistently higher than that of submucosal vessels. This difference may represent local regulation by either blood-borne agonists activating endothelial receptors, by shear stress caused by increased blood flow, or by low oxygen tension [47; 48]. In line with progressive neovascularization occurring in UC, mild disease caused no changes in the distribution of NOS-3, while an increased number of vascular profiles were evident in moderate UC. *Decrease in the intensity of vascular NOS-3 immunoreactivity in the lamina propria of UC specimens suggests a disturbance in the local regulation of blood flow.*

## NOS-2 CONTENT IN POUCHITIS CORRELATES TO THE CLINICAL DEGREE OF DISEASE

### *No-pouchitis group*

We compared the distribution of NOS-2 immunoreactivity in the terminal ileum of UC patients and in the ileal reservoir of the no-pouchitis group 4 to 86 months after the operation. By microscopic examination, occasional villi showed NOS-2-immunoreactive regions in both groups but the difference in NOS-2 immunoreactivity score was nonsignificant. In accordance, we obtained no significant differences in the acute inflammation scores between the control ileum and the no-pouchitis group. These observations indicate that construction of the reservoir per se does not induce significant NOS-2 expression.

### *Pouchitis groups*

Acute inflammation score based on neutrophil infiltration was increased in all pouchitis groups. Villus atrophy in all pouchitis groups was observed here, as well as in previous studies [127; 190]. These findings confirm that acute inflammation was in progress in all three pouchitis groups studied here, although the clinical course and the symptoms were different in each group.

Like in UC, we demonstrated that NOS-2 immunoreactivity is also induced in the epithelium of the inflamed ileal reservoir. NOS-2 immunoreactivity score correlated significantly with acute inflammation score based on neutrophil infiltration. NOS-2 immunoreactivity also correlated in the present study with clinically estimated severity of pouchitis. Although induction of NOS-2 is not specific for UC [26; 45; 46; 191], changes in NOS and substance P in UC and pouchitis suggest similarities in pathogenesis of the two diseases*. Taken together, our results suggest that NOS-2 immunoreactivity provides a useful marker for pouchitis. NOS-2 would be a more accurate indicator of pouchitis than substance P, since the latter remains elevated irrespective of clinical improvement of pouchitis* [126].

## NOS-2 INDUCTION IN POUCHITIS AS A DEFENCE MECHANISM

Inducible NOS in pouchitis may have protective functions in the injured ileal epithelium. This hypothesis is supported by analogy from observation on UC. Although direct conclusions regarding human UC cannot be drawn, a similar protective role is feasible, since NOS-2 immunoreactivity was conspicuously localized in the apical epithelium both in human colitis and in pouchitis.

The evidence for a role of bacterial invasion in the pathogenesis of UC or pouchitis is contradictory. Continent pouch contains more extensive bacterial growth than normal ileum content, and pouchitis responds readily to antimicrobial therapy [192; 193]. Intramural bacteria have been described electron microscopically in 47 % of patients with a pouch, irrespective of whether the

patients developed pouchitis [194]. In contrast, no bacterial invasion was reported in UC patients, while increased bacterial growth was observed on the surface mucus [186] and mucosal IgG antibodies against commensal enteral bacteria are increased in UC [195]. Enteroinvasive bacteria are known to induce directly NOS-2 expression in human colon epithelial cells and to result in NO production primarily in the apical part of the epithelial cell [196]. Increased NO generation caused by cytokines, bacterial products or mitogens has also been reported in cultured small intestinal epithelial cells [197]. *Taken together, increased expression of NOS-2 in relation to the severity of pouchitis may represent activation of epithelial NOergic defense mechanism against bacterial invasion in pouchitis.*

Permeability of the mucosa is increased in pouchitis [198]. Increased intestinal permeability after thermal injury is correlated with induced NOS-2 activity, which is localized mainly in enterocytes and which is prevented by S-methylthiourea, a specific NOS-2 inhibitor [135]. Nitric oxide donor induces hyperpermeability of cultured intestinal epithelial monolayers [188]. *These observations suggest that NO production by the induced NOS-2 observed in the present study may be the cause of increased permeability in pouchitis.*

## NOS-3 IN POUCHITIS

Endothelial NOS immunoreactivity readily revealed the vasculature in the lamina propria, thus the increase in the number of vascular profiles was expected in pouchitis specimens. While the villus core in the normal ileum and no-pouchitis groups only rarely showed NOS-3-immunoreactive vascular profiles, such profiles were common in the pouchitis groups (3-5). This is surprising, since the endothelium is thought to express NOS-3 uniformly. Appearance of NOS-3-immunoreactive vessels in the villus core in pouchitis might represent increased NOS-3-acitivity and/or vascular proliferation due to inflammation. A less likely explanation is that NOS is induced by pouchitis in villus endothelium not normally expressing it. Increased NOS-3 immunoreactivity in pouchitis may reflect changes in pouch microcirculation.

## NERVE TERMINALS CONTAINING NEUROPEPTIDES DECREASE IN NUMBER AFTER MASSIVE SMALL BOWEL RESECTION

After massive small bowel resection, the remaining intestine undergoes both morphological and functional changes to obtain sufficient absorption of nutrients. [29-31; 144]. The mechanisms for the marked motor and secretory changes that follow small bowel resection are largely unknown.

Massive small bowel resection caused a significant decrease in the number of VIP-immunoreactive nerves in the mucosa and the circular muscle. This is in accordance with the findings on reduced VIP tissue concentration in dogs subjected to massive resection of the small bowel [159]. In the rat, massive small

bowel resection caused an increase in the number of VIP-immunoreactive neurons in the submucosal ganglia, and this change was accompanied by increased ganglion and neuron size [158]. Nerve fibers were not investigated in that study. In the light of the present study, this observation probably represents increased VIP synthesis in such interneurons, which do not project outside the ganglia and which normally express no or only very low levels of VIP.

VIP is a potent stimulant of intestinal secretion [86]. It acts directly via VIP receptors activating production of cAMP in enterocytes to increase intestinal secretion. However, rat small intestine shows decreased affinity of VIP receptors after resection [199]. This observation, together with the present result, predicts that VIP-induced secretion is impaired after resection. Moreover, it is consistent with the known inhibitory role of cAMP on cell proliferation in the epithelium and suggests participation of VIP in the compensatory hyperproliferative response of the remaining intestine following massive resection of the small intestine.

Normally VIP-containing nerve terminals contribute to the peristaltic reflex so that their projections mediate descending inhibition of the reflex, while substance P contributes to the stimulatory effect. Extensive proximal resection causes a decrease in the motility of the remnant intestine, resulting in compensatory improvement of absorptive function [156; 157]. Our finding that the density of VIP-immunoreactive (inhibitory) nerve fibers decreases while that of substance P-immunoreactive (stimulatory) fibers remains unchanged would predict the opposite.

In the pig, progressive decrease in plasma levels of VIP has been measured after massive small bowel resection [200]. In accordance, the portal release of VIP is inhibited in resected gut in the dog [201]. The decrease in the number of VIP-immunoreactive fibers observed in this study may be related to decreased plasma concentration of VIP by diminished secretory activity of VIP neurons. In contrast, human plasma levels of VIP are elevated in short bowel syndrome. Several factors have been suggested to contribute to this: an increased gastric load in the residual bowel, a compensatory increase in blood supply to the gut, removal of an inhibitory factor arising in the small intestine, or mucosal stress of the colon due to unabsorbed food [202].

The resection failed to induce any quantitative changes in galanin immunoreactivity in the mucosa. Galanin inhibits, in part, the release of hormones in the gastrointestinal tract. Postprandial enteroglucagon and PYY plasma concentration decrease during galanin infusion and increase after resection [203; 204]. Lack of resection-induced changes in galanin immunoreactivity in the mucosa suggests that enteroglucagon and PYY levels are only partially regulated by galanin. However, the number of galanin-immunoreactive nerves in the muscle layer decreased markedly after the resection. Galanin inhibits intestinal smooth muscle contractility in the human

and in several animal species [203; 205]. Thus, the effect of galanin in resected gut is synergistic with that of VIP.

Enkephalin-immunoreactive nerves were observed only in the muscle layer. In the 5-month-old control pigs, we observed significantly more enkephalin-containing nerves than in 3-month-old piglets. On the other hand, the distribution of enkephalin in the 5-month-old operated piglets was similar to the 3-month-old-old control piglets. Thus the increase in the density of enkephalin innervation during natural development is inhibited by the resection. Enkephalin is one of the opioid peptides that have numerous and complex effects on gut motility, such as delaying whole gut transit time and gastric emptying and inhibition of gastric contraction. Thus, the well-known clinical effect of opioids in treatment of diarrhea suggests that one physiological function of the endogenous opioid peptides is to limit the intensity of intestinal excitation and thus decelerate gut motility [70]. The present observation on the decreased number of enkephalin-immunoreactive nerve fibers in the remnant gut as compared to the age-matched control indicates that enkephalin-mediated effects are decreased due to the resection. This parallels with alteration in VIP-and galanin-mediated effects. Hence, all three neuropeptides have inhibitory effects on the intestinal motility and resection diminishes these effects. The mechanisms of the overall decrease in the motility after proximal resection, as reported in previous studies, remains unknown [156; 157]. However, the reduction of inhibitory neuropeptides may reflect changes in propulsive and mixing peristalsis in short bowel syndrome.

The distribution of substance P and somatostatin-immunoreactive nerves was similar in all three groups. Although minute changes may be missed by visual estimation, our observation suggests that these two neuropeptides do not essentially contribute to the changes induced by resection. Previous studies give only fragmentary information on their effects. An increase in the size of submucosal somatostatin-immunoreactive neurons after massive small bowel resection in the rat has been reported [158]. In contrast, in the dog no change in somatostatin tissue concentration in the remaining small intestine was found after massive distal resection [159].

*The results of our study suggest that massive resection induces significant changes in the neuropeptide-containing innervation in the mucosa and muscle layer. The changes are specific for VIP, galanin, and enkephalin and most probably contribute to altered motor activity and altered mucosal function in the remaining intestine.*

## *CONCLUSIONS*

1. GAP-43 is a universal neuronal marker in the mature human enteric nervous system. GAP-43 immunoreactivity reveals more numerous and thicker nerve fibers than PGP 9.5, synaptophysin, or NSE immunoreactivities although the true staining intensity is essentially similar for all these markers. In contrast, NSE is a superior marker of neuronal somata.

2. The present study corroborated the special role of substance P in UC. Ulcerative colitis does not significantly change the total number of nerve fibers in the colon. In contrast, the density of substance P-containing nerve terminals increases specifically This occurs exclusively in the mucosa, and is likely due to increased peptide synthesis leading to better visibility of the nerve fibers.

3. The present study demonstrates that NO affects the function of colon by several mechanisms: Neuronal NOS (NOS-1) disappears selectively from the nerves of muscularis mucosae of UC colon. Inducible NOS (NOS-2) content increases in the epithelium of UC colon in relation to the severity of the disease. Ulcerative colitis causes an increase in the number of vascular profiles, and a relative decrease in the level of endothelial NOS (NOS-3) in the lamina propria.

4. Expression of inducible NOS correlates with both the clinical degree of pouchitis and with the severity of acute inflammation.

5. Massive resection induces significant changes in the neuropeptide-containing innervation in the mucosa and muscle layer. The changes are specific for VIP, galanin, and enkephalin and are compatible with altered motor activity and altered mucosal function in the remaining intestine.

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